ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

VOL. 31 1953 FASC. 4

MERCATORIN KIRJAPAINO
HELSINKI, FINLAND

Annales Medicinae Experimentalis et Biologiae Fenniae

is a direct continuation of the *Acta Societatis Medicorum Fennicae* »*Duodecim*», 1919—1930 (Vols. I—XII) and the *Acta Societatis Medicorum Fennicae* »*Duodecim*», *Ser. A*, 1931—1946 (Vols. XIII—XXIV).

The journal is published by the Finnish Medical Society »Duodecim» with the object of providing an opportunity to publish articles on experimental medicine and on related biological subjects.

Each number of the journal contains 80—100 pages, four numbers forming one volume. Articles are accepted for publication with the understanding that they are original contributions never previously published. The manuscripts should be in English, French or German, and typewritten. They should not exceed a total length of 16 pages, and there should be a short summary at the end of the article.

The subscription price is Fmks 750 per volume in Finland and $ 4 or Swedish Crowns 15.00 in foreign countries. More extensive works, published as supplements, the subscribers receive free of charge.

Address for subscription, exchange of reviews, and all communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finland.

Annales Medicinae Experimentalis et Biologiae Fenniae

est une suite directe des revues *Acta Societatis Medicorum Fennicae* »*Duodecim*», 1919—1930 (V. I—XII) et *Acta Societatis Medicorum Fennicae* »*Duodecim*», Ser. A, 1931—1946 (V. XIII—XXIV).

La revue est éditée par la Société de médecins finnois »Duodecim», et a pour but d'offrir l'occasion de publier des recherches scientifiques appartenant à la médicine expérimentale et à la biologie étroitement liée avec la médicine.

La revue paraît en cahiers comprenant à peu près 80—100 pages. Quatre cahiers forment un volume. Les articles destinés à la revue ne doivent pas être publiés ailleurs. Les manuscrits dactylographiés doivent être rédigés en français, allemand ou anglais et leur longeur totale ne doit pas en général dépasser 16 pages. Un court résumé doit se trouver à la fin.

Le prix de l'abonnement pour la Finlande est marcs 750, pour l'étranger $ 4 ou couronnes suédoises 15 par volume. Les travaux plus étendus, qui seront éventuellement publiés en suppléments, seront distribués gratuitement aux abonnés.

Adresse pour abonnement, échange de journaux et toutes autres communications:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finlande.

Annales Medicinae Experimentalis et Biologiae Fenniae

sind eine direkte Fortsetzung der *Acta Societatis Medicorum Fennicae* »*Duodecim*», 1919—1930 (Vol. I—XII) und der *Acta Societatis Medicorum Fennicae* »*Duodecim*», *Ser. A*, 1931—1946 (Vol. XIII—XXIV).

Die Zeitschrift wird vom *Finnischem Ärzteverein* »*Duodecim*» herausgegeben und hat zur Aufgabe wissenschaftliche Untersuchungen aus dem Gebiete der experimentellen Medizin und sich daran schliessenden biologischen Forschungsgebieten aufzunehmen.

Jede Nummer der Zeitschrift erscheint in einem Umfange von 80—100 Druckseiten. Vier Nummern bilden ein Volumen. In die Zeitschrift werden nur Originalarbeiten aufgenommen, die nicht früher veröffentlicht worden sind. Die mit Schreibmaschiene geschriebenen Manuskripte sind in deutscher, englischer oder französischer Sprache einzusenden und sollen im allgemeinen nicht mehr als 16 Druckseiten betragen. Jede Untersuchung ist durch eine kurze Zusammenfassung abzuschliessen.

Der Bezugspreis beträgt für das Innland 750 Fmk und fürs Ausland 4 $ bzw 15 Schwedenkr. Umfangreichere Untersuchungen, die als Supplemente herausgegeben werden können, erhalten die Abonnenten abgabefrei.

Bezugsadresse sowie Anschrift für Austausch von Zeitschriften und alle anderen Mitteilungen:

Annales Medicinae Experimentalis et Biologiae Fenniae

Yrjönkatu 17, Helsinki, Finnland.

FROM THE DEPARTMENT OF BACTERIOLOGY AND SEROLOGY, UNIVERSITY OF TURKU

INFECTIONS CAUSED BY SALMONELLA MONTEVIDEO AND THE BACTERIOLOGICAL IDENTIFICATION OF THIS STRAIN

by

J. A. GRÖNROOS

(Received for publication February 9, 1953)

Salmonella montevideo was first isolated from lymph nodes of pigs by Hormacche and Peluffo (11) in Uruguay in 1936. They then discovered it in specimens taken from patients with acute infections, especially from children with summer diarrhoea (11, 12), and in specimens taken from animals. *S. montevideo*, like other strains causing diarrhoea, is also found in birds (13). Outbreaks attributed to raw eggs and egg powder have been reported (5, 24). Edwards & Bruner (4) reported in 1943 that among 2285 Salmonella strains they detected 21 of *S. montevideo* and in 1948 that among 1677 outbreaks 96 were caused by *S. montevideo*. Seligman *et al.* (23), of the Salmonella Center, New York, reported that in 1939—1945 altogether 2916 Salmonella strains, representing 54 different types, had been diagnosed at their institute; of these 134 were *S. montevideo* strains. At the Swedish State Bacteriological Laboratory 1152 strains were diagnosed in 1936—1942 (20). One of them was *S. montevideo*. In 1936—1940 there were 373 outbreaks of diarrhoea in Denmark (9), with only 2 sporadic cases of *S. montevideo*. Since then no outbreak caused by *S. montevideo* has been reported in Denmark (15). In the American 15*th* Medical General Laboratory, functioning during World War II (1943—1945) at Naples as the laboratory for troops taking part in the Mediterranean campaigns, 892 Salmonella strains were found, of which 115 were *S. montevideo* (2), Specimens had not only been sent to

the laboratory from the American troops; there were also specimens taken from soldiers of other allied nations, from civilians and war prisoners.

The infections caused by *S. montevideo* reported by Edwards, Seligman *et al.* were, as a rule, of a gastro-enteric character, but there were also cases of septicemia. In Seligman's series there was one fatal case. It may be mentioned that *S. montevideo* has been isolated not only from faeces and urine specimens, but also from local inflammations, such as those of the knee-joint (7) and of the lungs, from cervical pus (23), from the bile (four cases) (4), and several times from the blood (4, 5, 23).

The so-called food poisonings are also considered as being of a gastro-enteritic character. They are often caused by Salmonella. Outbreaks of food poisoning caused by *S. montevideo* have also been reported (1, 3, 4, 18, 24).

CASES REPORTED IN FINLAND

Apart from the common *S. paratyphi B.*, *S. typhi murium* and *S. typhi*, the following Salmonella types have been identified in Finland: *S. dublin* (19), *S. enteritidis* (10), *S. enteritidis var. danyz* (17), *S. paratyphi A* (16) and *S. abortus equi* (22). In addition, *S. cholerae suis var. Kunzendorff* has been isolated from animals (22). In recent years, several infections caused by *S. montevideo* have been detected, viz., two at the State Serum Institute (21) and six, since Jan. 1st, 1951, at the Department of Bacteriology and Serology of the Turku University. At this latter department 428 cases of infections caused by *S. paratyphi B.*, 28 caused by *S. typhi*, 21 caused by *S. typhi murium* and 3 caused by *S. enteritidis* have been diagnosed since 1951. Thus the frequency of the occurrence of *S. montevideo* appears to be the same as in the series referred to above.

In this country infections caused by *S. montevideo* have not previously been described. The following are reports on six cases.

Case 1. — A 37-year-old widow, a machine weaver, was sent to the University Hospital of Turku on Feb. 22, 1951, after having suffered from diarrhoea and fever during 10 days. Owing to her complaint of diffuse abdominal pains, she was gynecologically examined. As this examination proved to be negative, she was transferred to the Epidemic Hospital

with the diagnosis paratyphoid(?). The patient had no fever and except for the tenderness around the umbilicus, there were no symptoms. The blood count was normal except that eosinophils were absent. Her urine contained no albumen. A gram-negative rod was isolated from specimens of faeces and urine taken on Feb. 23 and Feb. 26. According to the biochemical reactions, the strain belonged to the Salmonella group. The Widal test was negative. The patient was discharged on Feb. 26 before the bacteriological diagnosis was completed.

Case 2. — A farmer aged 30 was sent to the Epidemic Hospital on Aug. 6, 1951, with the diagnosis paratyphoid. This patient had suffered 10 days from a slight temperature, headache and abdominal pains. On admission to the hospital, the patient had no temperature and was free from any symptoms. No signs of infection were evident in the blood picture. A Widal test conducted on Aug. 7 was negative. *S. montevideo* was isolated from the faeces specimen taken on the same day. The patient was discharged from the hospital on Aug. 9 before the final bacteriological diagnosis was made.

Case 3. — A sales manager of 28 became ill with a high fever on July 4, 1952, after having suffered from diarrhoea two days. As the patient complained of abdominal pains upon pressure at McBurney's point, the county medical officer sent him to the hospital on July 5. The patient had a high temperature during three days, but had no fever on the fourth day. Except for tenderness upon pressure the patient had no symptoms. He was given streptomycin and phtalazole. *S. montevideo* was isolated from the stools and the blood. The Widal test was negative. During convalescence, the patient continued to excrete the causative organism, and although he had no symptoms, *S. montevideo* was isolated twice from the blood. Samples of bile taken on the third and seventh week after the patient had fallen ill gave negative results for *S. montevideo.* During the seventh week of illness he was given 48 grams of chloromycetin. During this treatment no causative organism could be isolated from the urine and faeces, but a week after the treatment was terminated, the patient again excreted *S.montevideo* bacilli. He was discharged as a carrier on Sept. 2. Five samples of faeces and urine taken in January, 1953, gave no positive results on culture.

Case 4. — On July 31, 1952, a sailor, 30 years of age, was hospitalized with a high fever (ad 40° C) and diarrhoea that had begun on July 29. His stools were watery and he suffered from vomitings, periodic abdominal pains, headache and aching limbs. In the hospital the temperature of the patient varied from 38 to 49° during two days. Except for a diffuse tenderness of the abdomen upon pressure, he was free of symptoms. The blood picture showed leukopenia; eosinophils were absent. A blood culture made on Aug. 31 was negative. *S.montevideo* was isolated from both the faeces and urine. The Widal test was negative. The patient was transferred to the Epidemic Hospital. The patient continued to excrete *S.*

montevideo bacilli during one month and a half. He was discharged on Sept. 19.

Case 5. — A man of 63, who according to the anamnesis had suffered from food poisoning with diarrhoea and fever for one week in June 1952 was hospitalized in August with carcinoma intestini. *S.montevideo* was isolated from the faeces. The Widal test was negative.

Case 6. — According to the report of the county medical officer, a middle-aged woman had had diarrhoea and fever during two days. *S.montevideo* was isolated from the faeces. The woman remained a temporary carrier for two months.

All of these cases showed fever, abdominal pain, and, with the exception of the second case, diarrhoea. The fever and diarrhoea lasted from two to ten days. In all the cases, *S. montevideo* was isolated from the faeces, in the fourth case also from the urine, and in the third case three times from the blood. In the third, fourth and sixth case the patients excreted the bacillus during a longer period.[1] The fifth patient may have excreted this bacillus after his food poisoning which was probably caused by *S. montevideo.* In all cases the clinical features could be described as an infection similar to acute gastro-enteritis.

The cases were all sporadic: one was from Metsämaa, one from Oripää, one from Loimaa, one from Turku, one from Pori, and one from Alastaro. In the case of four patients, specimens were also taken from members of the patients' families and from persons living in the nearest neighbourhood. In none of the cases was the source of infection established.

BACTERIOLOGICAL DIAGNOSIS

Since 1951 the routine bacteriological examination of stool and urine specimens has been carried out at this laboratory in the following manner. The stool and urine specimens are cultured on the day of arrival, the former specimens directly on Difco SS-agar (primary plate) and the latter specimens on brom cresol purple plates containing 0.1 per cent sodium desoxycholate (brcpd) (8) and both on selenite enrichment medium.

[1] The patients were discharged after three successive specimens of faeces and urine taken after 2 to 3 day intervals were found free of bacilli; in the case of people handling foodstuffs, five negative tests are required before discharge.

The primary plates are tested on the next day and after 48 hours' incubation. The inoculated selenite broth is incubated overnight and from this a brcpd plate is seeded. Characteristic colonies are picked up with wire and inoculated into Difco Triple Sugar Iron agar (TSIA) tubes and into urea tubes. If urea decomposition occurs, no further analyses are made. If the reactions in the TSIA tubes are found to be typical of Salmonella strains, production of hydrogen sulphide, an alkaline slant, and a typical acid butt with or without gas, the strains are tested further by means of biochemical reactions and slide agglutination tests following the simplified technique of Kauffman and Edwards (14) for identifying Salmonella strains.

From the cases described above, the *S. montevideo* strains were isolated by the author by means of biochemical reactions and identified by performing agglutination tests. The strain 357/51

TABLE 1

SLIDE AGGLUTINATION TESTS WITH S.MONTEVIDEO TYPE STRAIN AND THE ISOLATED STRAINS

	O Immune Sera Containing Agglutinins for			
	VI, VII	VI, VIII	VII	I, II, XII
S. 46	+++	+++	+++	—
357/51 ..	+++	+++	+++	—
3030/51 ..	+++	+++	+++	—
4792/52 ..	+++	+++	++	—
6110/52 ..	+++	+++	+++	—
6523/52 ..	+++	+++	+++	—
7698/52 ..	+++	+++	++	—

	H Immune Sera Containing Agglutinins for					
	gm	gp	gms	mt	s	t
S. 46	+++	+++	+++	+++	+++	—
357/51 ..	+++	+++	+++	+++	+++	—
3030/51 ..	+++	+++	+++	+++	+++	—
4792/52 ..	+++	+++	+++	++	+++	—
6110/52 ..	+++	+++	+++	+++	+++	—
6523/52 ..	+++	+++	+++	+++	+++	—
7698/52 ..	+++	+++	+++	++	+++	—

TABLE 2

ABSORPTION AND AGGLUTINATION TESTS WITH O AND H IMMUNE SERA OF SALMONELLA MONTEVIDEO TYPE STRAIN S. 46 AND STRAINS NOS. 3030/51 AND 4792/52

S. paratyphi A has been employed as a negative control strain

Immune Sera	Absorbed with Strains	Titrated with Strains			
		S. 46	3030	4792	S. parat. A
O S.46		640	320	1 280	0
	46	0	0	0	0
	3030	0	0		0
	4792	0		0	0
	S. parat. A	320	320	640	0
O 3030		320	320		0
	46	0	0		0
	3030	0	0		0
	S. parat. A	160	160		0
O 4792		320		320	0
	46	0		0	0
	4792	0		0	0
	S. parat. A	320		160	0
H S.46		51 200	51 200	12 800	0
	46	0	0	0	0
	3030	0	0		0
	4792	0		0	0
	S. parat. A	25 600	12 800	25 600	0
H 3030		51 200	51 200		0
	46	0	0		0
	3030	0	0		0
	S. parat. A	12 800	25 600		0
H 4792		12 800		12 800	0
	46	0		0	0
	4792	0		0	0
	S. parat. A	6 400		12 800	0

isolated from case 1 was kindly identified by Dr. F. Kauffman, Chief of the International Salmonella Centre.

The isolated strains possessed the following bacteriological properties. All strains were motile and fermented glucose, arabinose, sorbitol, dulcitol, maltose, mannitol, rhamnose, trehalose and

xylose. They were not able to ferment adonitol, salicine, lactose, and sucrose during 30 days' incubation. The strains 3030/51 and 7698/52 were inositol positive variants, but the other strains were unable to ferment inositol. The strains produced no indole. The reactions with methyl red were positive, the Voges-Proskauer and urea tests negative. All strains produced hydrogen sulphide and utilized citrate (Simmons). They also reduced nitrate. They were not able to liquefy gelatin during a period of 30 days. According to these reactions the strains belong to the Salmonella group.

By slide agglutination tests conducted with immune sera, some of them absorbed (Table 1), the strains were found to have the following O and H antigens: VI, VII: *g, m, s.*

In two cases the bacteriological diagnosis was confirmed by preparing O and H immune sera with a *S. montevideo* strain type S. 46 and with two of the isolated strains Nos 3030/51 and 4792/51. The immune sera were absorbed and titrated (14) as shown in Table 2. As seen from the table, the strains are capable of exhausting each other's serum, and hence the isolated strains have the same antigens VI and VII and g, m and s in common with *S. montevideo.* It was confirmed that none of the six isolated strains possesses a second phase by inoculating swarm agar plates containing pure g, m, s immune serum at a dilution of 1: 100, whereupon the strains were precented from swarming. It is thus obvious that the type strain and the strains 3030/51 and 4792/52 are identical.

SEROLOGICAL DIAGNOSIS

As usual, the Widal test is carried out with antigens of *S. paratyphi B., S. typhi,* and *S. typhi murium.* It is therefore readily understood that the tests were negative in the present cases. When the sera of the patients were retested using *S. montevideo* antigens, the agglutination reaction was positive in the fourth case at a dilution 1: 2560 and in the fifth case at a dilution of 1: 320. This confirms the assuption that the food poisoning of the fifth case was due to *S. montevideo.* In the second case only a weak 0 titer (1: 80) was obtained. In the three other cases the Widal test remained negative even when it was conducted with the *S. montevideo* antigens.

COMMENT

It is obvious from the results described above that a Widal test is not alone sufficient when attempting a diagnosis of infections due to Salmonella strains. The Widal test may be positive and even with a high titer as a result of vaccination alone, but may also be negative in severe infections. For the diagnosis of typhoid, paratyphoid, dysentery, gastroenteritis and acute infections in general it is of importance to make cultures from the blood, faeces and urine of untreated patients since, to quote Seligman et al. (23), any Salmonella is capable of producing enteritis as well as septicemia, a typhoid-like syndrome, a local infection or a carrier state. Only in this way is it possible to identify unusual Salmonella strains which are also found along with the common Salmonella types.

SUMMARY

1. Six cases of acute infections caused by *S. montevideo* which resemble cases of gastro-enteritis are presented.

2. The serological and bacteriological diagnosis of the isolated strains is described.

3. In suspected cases of typhoid, paratyphoid or gastroenteritis or in certain acute infections the Widal test alone is not sufficient for the diagnosis of Salmonella infections. For determining the etiology of these diseases, cultures from Widal coagula or blood and from faeces and urine of untreated patients are of first — rate importance.

REFERENCES

1. Brandis, H.: Ztschr. Hyg. 1947:*127:*259.
2. Bruner, D. W., and Joyce, B. J.: Am. J. Hyg. 1947:*45:*19.
3. D'Albora, J. B., Ingegno, P. A., and Edson, J. N.: J.A.M.A. 1945: *129:*10.
4. Edwards, P. R., and Bruner, D. W.: J.Infect. Dis. 1943:*72:*58.
5. Edwards, P. R., Bruner, D. W., and Moran, B. A.: Ibid. 1948: *83:*220.
6. Gard, S.: Ztschr. Hyg. 1938:*120:*615.
7. Gordon, S. H., Hoffman, S. J., Schult , A., and Lomberg, F.: J.A.M.A. 1949:*141:*460.
8. Grönroos, J. A.: Ann. Med. Exper. et Biol. Fenniae 1950:*28:*20.
9. Harhoff, N.: Zentralbl. Bakt. Orig. 1941:*147:*194.

10. Hirvisalo, K. F.: Acta Soc. Med. Fennicae Duodecim 1933:*16:*1.
11. Hormacche, E., Peluffo, C. A., and Aleppo, P. L.: Ztschr. Hyg. 1937:*119:*453.
12. Hormacche, E., Suraco, M., Peluffo, C. A., and Aleppo, P. L.: Am. J. Dis. Child. 1943:*66:*539.
13. Jungherr, E., and Clancy, C. F.: J. Infect. Dis. 1939:*64:*1. Quoted in Zentralbl. Bakt. Refer 1940:*135:*384.
14. Kauffmann, F.: Enterobacteriaceae. Ejnar Munksgaard Copenhagen 1951.
15. Kauffmann, F.: Personal communication.
16. Kokko, U. P.: Acta Med. Scandinav. 1945:*167:*123.
17. Kokko, U. P.: Nord. med. 1947:*36:*2325.
18. Meyers, W. A., and Eckles, L. E.: U. S. Naval med. Bull. 1944:*43:* 1067.
19. Mustakallio, E.: Acta Soc. Med. Fennicae Duodecim 1943:*33:*53.
20. Olin, G., and Hammarström, E.: Nord. med. 1943:*18:*704.
21. Rantasalo, I.: Pikkulasten ripuleista (Diarrhoea of Early Childhood; Finnish). A paper read at a meeting of the Duodecim Society on March 6, 1952.
22. Rislakki, V.: Suomen Eläinlääkärilehti, 1945:*3:*11.
23. Seligman, E., Saphra, J., and Wassermann, M.: J. Immunol. 1946: *54:*69.
24. Watt, J.: Pub. Health Rep. 1945:*60:*835.
25. Vink, H. H., Scholtens, R. Th., and van Hees, C. A.: Nederl. tijdschr. geneesk. 1943:*87:*1433.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

THE ACTION OF FUNGUS EXTRACTS ON YEAST NUCLEIC ACID

by

REINO MÄKITALO

(Received for publication June 12, 1953)

In 1920 Jones (10) described a thermostabile enzyme present in the pancreas and capable of digesting yeast nucleic acid (RNA). The enzyme was purified by Dubos and Thompson in 1938 and crystallized in 1940 by Kunitz (15) who named it ribonuclease.

Soluble extracts of different animal tissues have been obtained which exhibit a high degree of enzymatic activity upon yeast nucleic acid. The distribution of the enzyme in the tissues has been recorded by numerous investigators (5).

Similar activity was shown by a variety of micro-organisms. Mc Fadyen (6) reported that Bacillus subtilis can depolymerise yeast nucleic acid, and Woodward (22) studied the ribonuclease activity of Pasteurella pestis. Miller et al. (21) studied the nucleic acid metabolism of malarial parasites.

Since I have found no data on the occurrence of those enzymes in plants, my intention here has been to study their occurrence in Finnish fungi.

PRESENT INVESTIGATION

The fungi for the investigation were collected from the surroundings of Helsinki (Regio Nylandia). They were identified by Mr. N. Malmström, M. Sc. Part of the fungi was dried at 37° C and kept in storage at room temperature. Part of them was kept fresh in a deep-freeze at —20° C.

Before the investigation the fungi were ground by hand in a mortar into a fine powder and a 1:10 extract was made in saline. The suspension was allowed to stand for an hour at +37, after which it was centrifuged for 10 min. at 3500 RPM. The solution obtained was passed once through ordinary filter paper and subsequently through Chamberlain filters. The completely clear liquid obtained was employed in a number of the investigations (Table 1).

After Chamberlain filtering two fungus extracts (Armillaria mellea and Clitocybe infundibuliformis) which had proved highly active were purified further by adding a five fold quantity of cold 94% alcohol to the clear liquid. The solution was allowed to stand overnight in the refrigerator after which the precipitation was separated by centrifuging (10 min., at 4500 RPM). The precipitate was redissolved in distilled water and the clear solution lyophilized. A pulverous highly active fungal substance was obtained. Deep-frozen it's biological activity was unaltered during the 4 months it was *tested*. A 1.0 % solution of this substance was employed in a number of the investigations (Fig. 1, 2, 3, 4).

METHODS

The ribonucleic acid (RNA, from British Drug Houses Ltd.) was purified by reprecipitating the commercial preparation with glacial acetic acid (1). The veronal acetate buffer was made according to Michaelis by which method a constant ion strength and isotonicity in relation to blood can be achieved. (19). For a number of experiments we employed an enzyme solution obtained by extracting 1 part of fresh fungus with 9 parts of saline and by filtering the solution through Chamberlain filters. In the other experiments the above enzyme solution prepared by the Freeze-Drying method was employed.

In order to test the ribonuclease activity we used the modified Davidson-Waymouth acid precipitating method (4): 4 ml of Mc Fadyen's uranylacetate-trichloracetic acid was added to the enzyme-RNA tubes after 18—24 hours digestion at 37° in a water bath. The enzyme-RNA tubes contained:

1.0 cc of ribonucleic acid 0.1 %
1.0 cc of veronal buffer *p*H 4.5
1.0 cc of enzyme solution
0.5 cc of 0.9 % NaCl solution

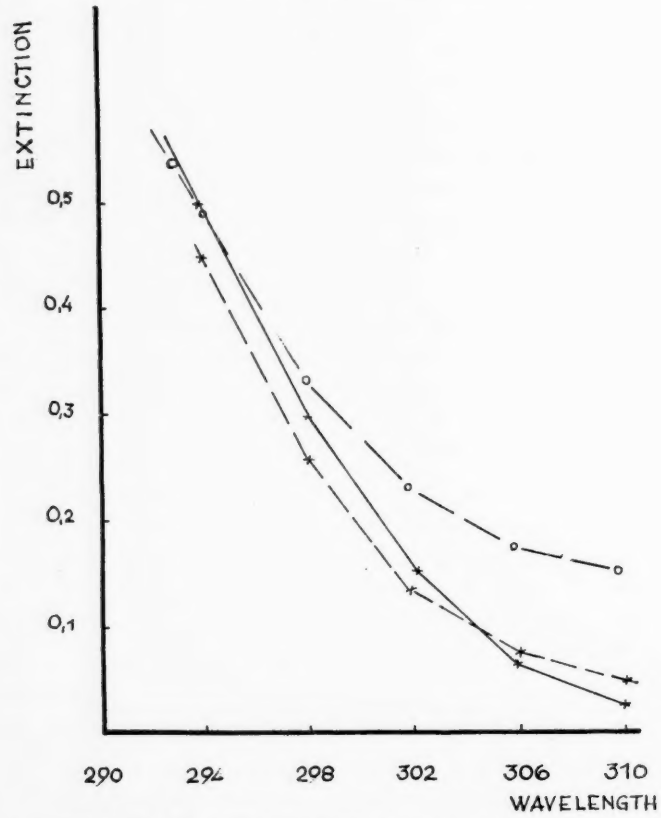

Fig. 1. — Effect of fungus extract on the extinction (density) of a solution of yeast nucleic acid in the range of 290—310 mμ at *p*H 4.5. Nucleic acid concentration 0.5 mg/ml.

———————: undigested yeast nucleic acid.
— — — — — — —: yeast nucleic acid digested with ribonuclease 0.0025 mg. per ml.
—o—o—o—o—o—o—: yeast nucleic acid digested with fungus extract 2.5 mg. per ml.

A quantitative determination of enzyme activity was also made by measuring the phosphorus from nonhydrolysed RNA (precipitated) colorimetrically by the Berenblum-Chain microdetermination method (2). The phosphomolybdenic acid was rapidly extracted into isobutyl alcohol and reduced by stannous chloride in HCl-solution. The aqueous layer was then separated and the extinction of the coloured isobutylalcohol layer was determined by EEL-photoelectric colorimeter with a suitable filter.

Efforts were also made to follow the ribonuclease action of the fungus enzyme by the Kunitz spectrophotometric method (16) determining the specific absorption of RNA in the ultraviolet absorption area (292—310 μm) before and after fungus enzyme action. The Beckmann method used was controlled by crystalline ribonuclease (from Mann Fine Chemicals, New York). However no move of the ultraviolet absorption band towards a shorter wavelength due to the ribonuclease of our fungus extracts was ascertainable (Fig. 1). That hydrolysis of RNA had nevertheless occurred was shown by the failure of uranylacetate-trichloracetic acid to precipitate the digested solution.

RESULTS

The ribonuclease activity of 130 fungus extracts was studied by the Davidson-Waymouth acid precipitating method. The enzyme solutions employed consist of filtered fungus extracts in saline. From the active extracts the activity was titrated. Table 1 gives the fungi investigated in alphabetic order, divided into four systematic groups.

Fig. 2 illustrates the strength of the activity of our two fungus extracts. The hydrolysis of 1 mg ribonucleic acid (1 ml 0.1 % solution) with titrated standard enzyme solution 0.0002 mg/ml and the titrated fungus extracts have been compared. The method of estimating the hydrolysis was that of Berenblum-Chain. It is apparent that the fungus extracts are roughly 4 times as active as the standard used. — The same fig. demonstrates also that the extract of armillaria mellea contains desoxyribonucleases in addition to ribonucleases. They were somewhat less active than its ribonucleases.

Fig. 3 shows the effect of *p*H on enzyme activity. The buffer solution was 1.0 ml of Michaelis's veronal acetate buffer *p*H 2.6—9.6. To 1.0 ml of 0.1 % buffered ribonucleic acid solution (1 mg) was added 1.0 ml of 1.0 % armillaria mellea extract for 15 min. at 37° C in a water bath, after which the enzyme effect was discontinued by uranylacetate-trichloracetic acid precipitation. A quantitative phosphorus analysis was made of the nonhydrolysed balance of ribonucleic acid. The enzyme proved active in the *p*H zone of 3.5—5.5, the optimum being *p*H$\sim$4.5.

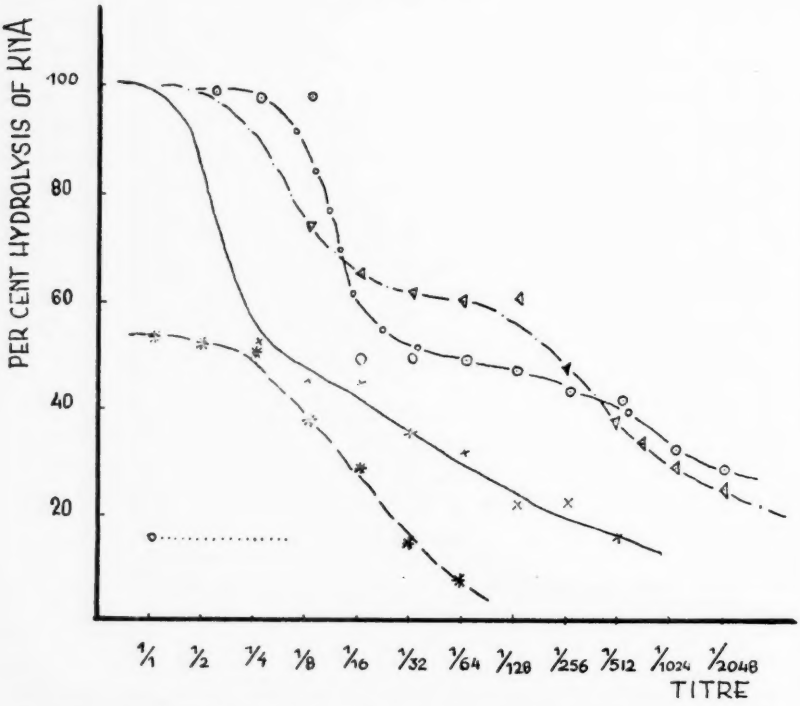

Fig. 2. — Hydrolysis of RNA with two fungus extracts and crystalline ribonuclease, with control, compared with the corresponding hydrolysis of DNA (18 hours, 37° water bath). Amount of ribonucleic acid: 1 mg (1 ml of 0.1 % solution). Amount of desoxyribonucleic acid: 1 mg (1 ml of 0.1 % solution). Ribonuclease concentration in the first tube 0.0002 mg per ml.

—·—Δ—·—Δ—·—: 1 % Armillaria mellea 1.0 ml + RNA
—o—o—o—o—o—: 1 % Clitocybe infundibuliformis 1.0 ml + RNA
———————— : crystalline ribonuclease solution + RNA
— — — — — — — : 1 % armillaria mellea 1.0 ml + DNA
. . . . . : fungus enzyme and uranyl acetate — trichloracetic acid reagent (4 ml) added simultaneously.

Fig. 4 shows the temperature optimum for the action of 1.0 % armillaria mellea (0.1 ml). 1 mg of RNA was subjected to the action by the enzyme solution buffered to *p*H 4.5. at different temperatures, for 15 and 5 sec. The reaction was discontinued by uranyl-acetate-trichloracetic acid precipitation and the nonhydrolysed ribonucleic acid was determined by the phosphorus analysis. The sudden cessation of hydrolysis above the temperature optimum at 70—80° C is probably due to inactivation of the enzyme (+80° C).

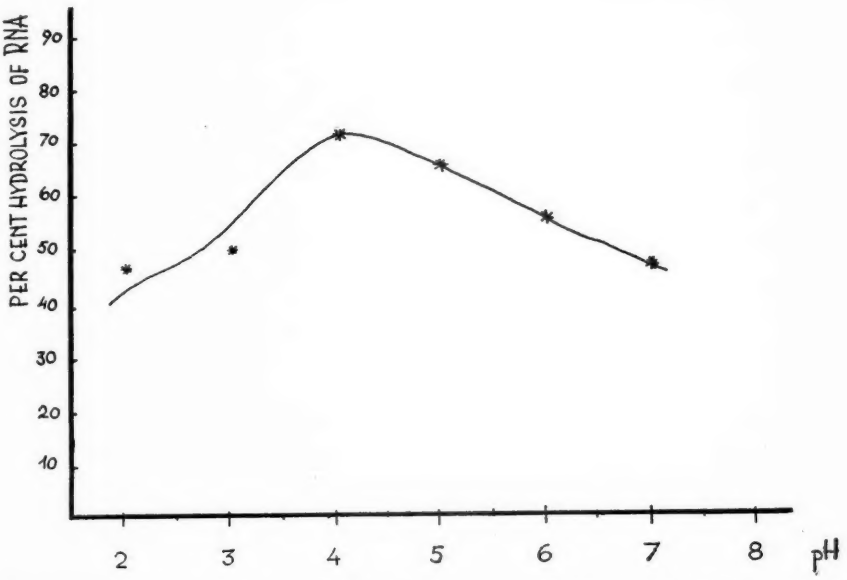

Fig. 3. — Effect of *p*H on the rate of digestion of yeast nucleic acid by fungus extract.

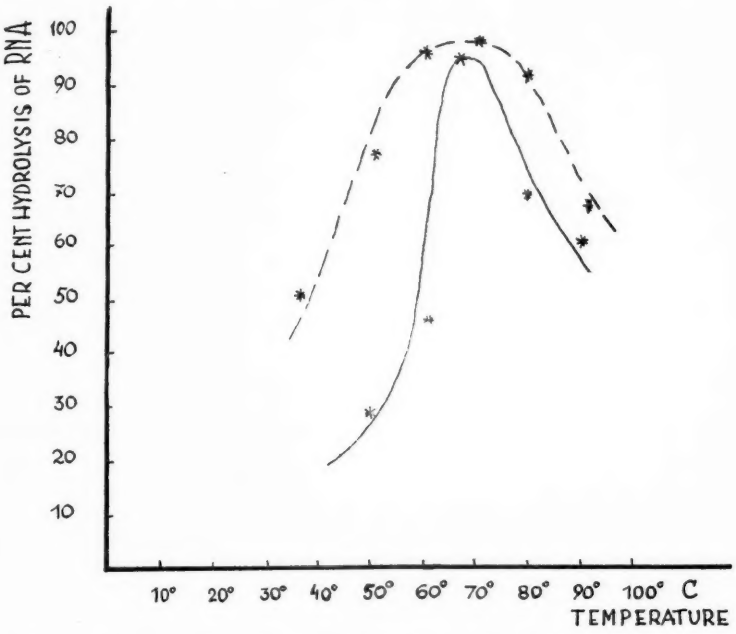

Fig. 4. — Effect of temperature on the rate of digestion of yeast nucleic acid by fungus extract.

———————: hydrolysis of 5 seconds.
— — — — — — — — : hydrolysis of 15 seconds.

TABLE 1

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly active (Titre 1/1—1/32)	Pronounced Activity (Titre ≥1/64)
HYMENOMYCETES		
Nomenclature principally according to Karsten (12, 13), supplemented with other authors listed in the bibliography (3, 9, 11, 14, 17, 18)		
	Amanita mappa (Batsch) Fr.	
	Amanita muscaria (L.) Fr forma aureola (Kalchbr.)	
	Amanita porphyria (Alb. & Schw.) Fr.	
	Amanita rubescens (Pers.) Fr.	
		Amanitopsis vaginata (Bull.) Roz.
		Armillaria mellea (Vahl.) Fr.
	Bjerkandera adusta (Willd.) Karst.	
Bjerkandera borealis (Fr.) Karst.		
Boletus badius (Fr.) Karst.		
	Boletus bovinus (L.) Karst.	
	» edulis Bull.	
	» felleus Bull.	
	» luteus L.	
	» scaber Bull.	
	» variegatus Schwarz.	
	» viscidus L.	
	Calocera viscosa (Pers) Fr.	
	Cantharellus auranthiacus (Wulf.) Fr.	
	Cantharellus cibarius Fr.	
	» tubiformis Fr.	
	» umbonatus (Pers.) Fr. = C. muscoides (Wulf.) Karst.	
	Clavaria fistulosa Fr.	
Clavaria ligula Schaeff.		
» flava Schaeff.		
Clitocybe clavipes (Pers.) Fr.		
	Clitocybe connata (Schum.) Fr.	

TABLE 1 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active (Titre 1/1—1/32)	Pronounced Activity (Titre ≥1/64)
	Clitocybe gilva (Pers.) Fr.	
	» infundibuliformis (Schaeff.) Fr.	
	» inversa Scop.	
	» nebularis (Batsch.) Fr.	
	» odora (Bull.) Fr.	
Clitopilus orcella (Bull.) Fr.		
	Collybia butyracea (Bull.) Fr.	
	» confluens (Pers.) Fr.	
	» dryophila (Bull.) Fr.	
	» maculata (Alb. & Schw.) Fr.	
	» platyphylla Fr.	
	» velutipes (Curt.) Fr.	
Coprinus comatus (Schum.) Fr.		
	Cortinarius alboviolaceus Fr.	
Cortinarius armillatus Fr.		
» camphoratus Fr.		
		Cortinarius cinnamomeus (L) Fr.
		» pholideus Fr.
		» sangvineus (Wulf.) Fr.
	Cortinarius semisangvineus Fr.	
		Cortinarius traganus Fr.
		» triumphans Fr.
	Creolophus corrugatus Fr.	
Flammula spumosa Fr.		
Fomitopsis connata Fr.		
» pinicola (Fr.) Karst. = Ungulina marginata (Fr.) Pat.		
	Gomphidius glutinosus (Schaeff.) Fr.	
Hansenia zonata (Fr.) Karst. = Coriolus zonatus (Fr.) Que'l.		

TABLE 1 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active (Titre 1/1—1/32)	Pronounced Activity (Titre ≥1/64)
	Hebeloma crustuliniforme (Bull.) Fr.	
	Hydnum corrugatum Fr.	
	» repandum L.	
	» rufescens Pers.	
	Hygrophorus agathosmus Fr.	
	» tephroleucus Pers.	
	Inocybe fastigiata Schaeff.	
	» geophylla (Sow.) Fr. var. alba. Lange.	
Laccaria laccata (Scop.) Cook. var. ametystina Bolt.		
	Lactarius camphoratus (Bull.) Karst.	
	» deliciosusus L.	
	» flexosus Fr.	
	» glyciosmus Fr. sensu Knauth & Neuhoff = L. confuses Lundell.	
	» helvus Fr.	
		Lactarius lignyotus Fr.
	» rufus (Scop.) Fr.	
	» subdulcis (Pers.) Fr. sensu Karst. Lundell etc. = L. thejogalus (Bull.) Fr. s. Knauth & Neuhoff.	
		Lactarius torminosus (Schaeff.) Fr.
	» turpis (Weinm.) Fr.	
	» trivialis Fr.	
	» vietus Fr.	
Lenzitina saepiaria (Schaeff.) Karst.		
	Lepiota acutesquamosa Weinm.	

TABLE 1 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active (Titre 1/1—1/32)	Pronounced Activity (Titre ≥1/64)
Lepiota amianthina (Scop.) Fr.		
	Lepiota clypeolaria (Bull.) Fr.	
		Lepiota cristata (Alb. & Schw.) Fr.
Lepiota granulosa (Batsch.) Fr.		
Marasmius oreades (Bolt.) Fr.		
	Marasmius peronatus (Bolt.) Fr.	
	» rotula Scop.	
	» scorodonius Fr.	
	Mycena galericulata (Scop.) Fr.	
	» epipterygia (Scop.) Fr.	
	» metata Fr.	
Mycena pura (Pers.) Fr.		
» vulgaris (Pers.) Fr.		
Naematoloma capnoides (Fr.) Karst.		
	Naematoloma sublateritium (Schaeff.) Karst.	
Paxillus atrotomentosus (Batsch.) Fr.		
Paxillus involutus (Batsch.) Fr.		
	Pholiota aurivella Batsch.	
Pholiota caperata Pers.		
» mutabilis (Schaeff.) Fr.		
	Piptoporus betulinus (Bull.) Karst. = Ungulina betulina (Bull.) Pat.	
Polyporus confluens (Alb. & Schw.) Fr.		
» ovinus (Schaeff.) Fr.		
» sulphurens Bull.		

TABLE 1 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active (Titre 1/1—1/32)	Pronounced Activity (Titre ≥1/64)
Polystictus Schweinitzii Fr. = Phaeolus Schweinitzii (Fr.) Pat.		
Polystictus perennis L.		
	Psalliota arvensis (Schaeff.) Fr.	
	» hortensis (Cooke.) Coll.	
	Russula aeruginea Fr.	
	» claroflava Grove.	
	» decolorans Fr.	
	» emetica (Schaeff.) Fr.	
	» foetans Pers.	
Russula fragilis (Pers.) Fr. = R.Mairei Singer Lange 1940		
		Russula integra Fr.
		» lutea Huds.
	Russula paludosa Britz = R. elatior Lindbl.	
		» vesca Fr.
	» xerampelina Schaeff.	
Stropharia aeruginosa (Curt.) Fr.		
» depilata (Pers.) Fr.		
	Tricholoma album (Schaeff.) Fr.	
	» flavobrunneum Fr.	
		Tricholoma equestre (L.) Fr.
Tricholoma rutilans (Schaeff.) Fr.		
	» virgatum Fr.	
GASTEROMYCETES		
Nomenclature according to Th. Fries (8)		
	Bovista nigrescens Pers.	
	» plumbea Pers.	

TABLE 1 (Cont.)

Name and Grouping of the Fungi		
Inactive	Active	
	Fairly Active (Titre 1/1—1/32)	Pronounced Activity (Titre ≥1/64)
Scleroderma aurantium Pers. (= Scl. vulgare Hern)	Lycoperdon pyriforme Pers. » umbrinum Pers.	
DISCOMYCETES Nomenclature according to W. Migula (20)		
	Otidea onotica (Pers.) Fuck.	
MYXOMYCETES Nomenclature according to Rob. Fries (7)		
	Lycogala Epidendrum (L.) Fr.	

SUMMARY

The ribonuclease activity of 130 fungal species of Finland was studied by the Davidson-Waymouth acid precipitation method. Activity was found in 73 % of the 1:10 saline extracts of fungi.

The decomposition products are no longer precipitable with Mc Fadyen's uranylacetate-trichloracetic acid.

Ultraviolet absorption measurements seem to justify the assumption that the hydrolysis of RNA with fungus extracts does not advance as far as with crystalline ribonuclease.

The fungus extracts were active in a wide *p*H area, with *p*H 4.5 as the optimum.

The extracts were inactivated at 80° C in 10 minutes.

The woiter is indebted to Mr. A. Louhivuori, M.Sc., for ultraviolet absorption measurements.

REFERENCES

1. Bain, J. A., and Rush, H. P.: J. Biol. Chem. 1944:*153:*659.
2. Berenblum, I., and Chain, E.: Biochem. J. 1938:*32:*295.
3. Bourdot, H., and Galzin, A.: Hyménomycétes de France I, Marcel Bry, Sceaux 1927.
4. Davidson, N. J., and Waymouth, C.: Biochem. J. 1944:*38:*375.
5. Dubos, R. J., and Mc Leod, C. M.: Proc. Soc. Exp. Biol. and Med.: 1937:*36:*696.
6. Mc Fadyen, D. A.: J. Biol. Chem. 1934:*107:*297.
7. Fries, Rob. E.: Sv. Botanisk Tidskrift, Sth. 1912:*6:*721.
8. Fries, Thore C. E.: Arkiv för Botanik, K. Sv. Vetenskapsakademien, Sth. 1921:*17:*9:1.
9. Ingelström, Einar: Svampflora, Nordisk Rotogravyr, Sth. 1940.
10. Jones, W.: Amer. J. Physiol. 1920:*52:*203.
11. Kallenbach, F.: Die Röhrlinge (Boletaceae), Die Pilze Mitteleuropas I, Werner Klinkhardt, Leipzig 1926—42.
12. Karsten, P. A.: Rysslands, Finlands och den Skandinaviska halvöns hattsvampar I—III, Bidr. t. känned. Finl. nat. och folk, 32 o. 37, Finska Vetenskaps Societen, Helsingfors 1879 o. 1882.
13. Karsten, P. A.: Kritisk öfversikt af Finlands Basidsvampar, Ibid. 48, 1889.
14. Knauth, B., and Neuhoff, W.: Die Milchlinge (Lactarii), Die Pilze Mitteleuropas II, Werner Klinkhardt, Leipzig 1935—45.
15. Kunitz, M.: J. Gen. Physiol. 1940:*24:*15.
16. Kunitz, M.: J. Biol. Chem. 1946:*164:*563.
17. Lange, J. E.: Flora Agaricina Danica I—V, Soc. Advanc. of Mycology and Danish Bot. Soc., Copenhagen 1935—40.
18. Lundell, Seth and Nannfeld: Fungi Exiccati Suecici, praesertim Upsaliensis Fasc. XV—XVI. Inst. Syst. Bot., Upsala 1939.
19. Michaelis, L.: Biochem. Zeitschr. 1931:*234:*139.
20. Migula, W.: Kryptogamen Flora von Deutschland, Deutsch-Österreich und der Schweiz, III.3 Teil, 2. Abt. Friedrich von Zezschwitz, Gera, R., 1913.
21. Miller, Z. B. and Kozloff, L. M.: J. Biol. Chem. 1947:*170:*105.
22. Woodward, G. E.: J. Biol. Chem. 1944:*156:*143.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI, AND THE STATE SERUM INSTITUTE, HELSINKI

STUDIES OF AGGLUTINATION IN RHEUMATOID ARTHRITIS

I

ATTEMPTS TO PURIFY THE FACTOR CAUSING AGGLUTINATION OF SENSITIZED ERYTHROCYTES

by

ODD WAGER[1] and EVA ALAMERI

(Received for publication July 20, 1953)

The extraordinary serological qualities peculiar to sera from patients with rheumatoid arthritis have been the subject of numerous studies. In recent years the interest of many workers has been focused on the capacity of many rheumatoid sera to strongly agglutinate red cells sensitized with a subagglutinating dose of the homologous antibody.

In a previous work (13) one of us studied the occurrence of this phenomenon within a large series of cases. With the technique used the phenomenon could be demonstrated in 61 per cent of typical cases of rheumatoid arthritis, whereas the rheumatic fever group showed a »positive» test in 3 per cent and the other control groups in 2 per cent or less. A number of other investigators have obtained results comparable to ours (1, 2, 8, 9, 14, 15). — Many authorities agree that the phenomenon possesses only a limited diagnostic value because of its inconstant and rather late occurrence in clinically typical rheumatoid arthritis. From a theoretical point

[1] Aided by a grant from the Sigrid Jusélius Foundation.

of view, however, the phenomenon is of great interest. In our previous study (13) we arrived at the conclusion that the serum factor responsible for the phenomenon may exert its effect by intensifying the agglutination of the antigen by its homologous antibody. This thermostable factor was called the »agglutination activating factor» (AAF), a designation originally employed by Waaler (12). On the basis of present knowledge it seems possible, but not necessarily true, that the AAF is a property of normal human serum, which is enhanced in rheumatoid arthritis. We showed that the AAF can be adsorbed to sensitized sheep cells and again released from them by elution at +56°C (13). Non-sensitized sheep cells do not adsorb the AAF.

The exact chemical nature of the AAF is unknown. In ammonium sulphate fractionation it has been shown to remain within the globulin fraction (12), and in electrophoresis it moves with the β-γ-globulins (10).

In the present work, fractionations of rheumatoid sera and normal sera were carried out by the ethanol fractionation techniques. The various fractions were tested for their AAF activity and protein concentration. Electrophoretic studies were carried out with the AAF active fractions. Furthermore, attempts were made to purify the AAF by means of absorption elution procedures.

MATERIAL AND METHODS

Sera. — The rheumatoid sera emanated chiefly from Heinola Sanatorium for Rheumatic Diseases and from the ward for rheumatic diseases in the Kivelä Hospital.[1] Considerable quantities of sera were required for fractionations. Therefore, pooled sera were used throughout the experiments.

Estimation of AAF Activity. — The technique described elsewhere (13) was followed with slight modifications. In this method, sheep cells sensitized with $\frac{1}{2} \times$ MAD (minimum agglutinating dose) of homologous rabbit anti-sheep cell serum are used. — In the case of sera previously absorbed with normal sheep cells, the agglutination titer for sensitized sheep cells is a direct measure of AAF activity. When non-absorbed sera are tested, the differential

[1] The writers wish to express their thanks to the chief of the Kivelä Hospital, Professor P. Soisalo, M.D., and the chief of the Heinola Sanatorium for Rheumatic Diseases, Mr. V. Laine, M.D., for the rheumatoid sera.

agglutination titer (DAT), *i.e.*, the denominator of the titer for sensitized cells divided by that of the titer for normal cells, reflects the AAF activity.

The serum precipitate fractions to be tested were dissolved in 0.15 M saline and diluted to the original volume.

Method of Electrophoresis. — An Antweiler apparatus was used. The runs were carried out in barbiturate buffer for fifteen minutes under conditions: ionic strength 0.12, pH 8.6, 1.8 mA, 40 V, protein concentration 1.5—2.0 per cent.[1]

Determination of Protein Concentration. — If not otherwise stated, the protein concentrations were determined by measuring the absorption at 280 mμ with the Beckman spectrophotometer, model DU. Extinctions varying from 7 to 15 were chosen for the different fractions according to their electrophoretic compositions. To check the rough values thus obtained, simultaneous protein determinations were carried out in one of the series by the micro-Kjeldahl method. The values given by the Beckman determinations proved to be reasonably accurate.

FRACTIONATION PROCEDURES

Fractionation Method A. — The serum was brought to conditions: vol. 1.1, ethanol 10 per cent, pH 6.3, ionic strength 0.14, temp. —3°C. The precipitate formed (prec.fr. I) was separated by centrifugation. The supernatant was brought to conditions: vol. 1.3, ethanol 25 per cent, pH 6.3, ionic strength 0.1, temp. —5°C. The precipitate formed (prec.fr.II) was separated from the supernatant (snt.fr. II) by centrifugation.

Fractionation Method B. — The serum was fractionated into fractions II, III and IV—V—VI according to Cohn (3). Fraction III was then brought to conditions: vol.1, ethanol 0, pH 6.3, ionic strength 0.001 phosphate, temp. 0°C. The supernatant fraction III-1 and the precipitate fraction III-2 were separated by centrifugation. — Fraction III-2 was brought to conditions: vol. 2, ethanol 0, pH 6.5, ionic strenght 0.01 phosphate, temp. 0°C. The precipitate fraction III-2-2 and the supernatant fraction III-2-1 were separated by centrifugation.

Fractionation Method C. — The serum was fractionated into

[1] The writers are indebted to Miss Ulla Fagerholm, State Serum Institute, for kindly performing the electrophoretic runs.

fractions C, D, F and G according to Deutsch (4). Fraction C was then brought to conditions: vol. 2, ethanol 20 per cent, pH 5.56, ionic strength 0.05 acetate, glycine 0.55 M, temp. —5°C. The precipitate fraction C-2 and supernatant fraction C-1 were separated by centrifugation.

Absorption Elution Procedures with Chicken Cell »Ghosts». — The chicken cell »ghosts» were prepared according to a method described by Dounce and Lan (5). For absorption the serum fraction was mixed with half of its volume of packed »ghosts», sensitized with ½ × MAD (minimum agglutinating dose) of a specific rabbit anti-»ghost» immune serum, the sensitization procedure being similar to that described elsewhere for the sensitization of sheep cells (13). The mixture was kept overnight in the refrigerator. The »ghosts» were separated by centrifugation at + 2—4°C (6 minutes at 1000—2000 RPM) and washed three times with buffered saline (pH 7.2), the volume for each washing being brought to the original level. The supernatants from the washings were retained. The »ghosts» were then resuspended to the original volume and the suspension kept for 5 minutes in a water-bath at +56°C. The »ghosts» were spun down quickly, and the supernatants (Eluate I) retained. At this stage the »ghosts» showed a tendency to form gelatinous clumps. The »ghosts» were resuspended as completely as possible to the original volume and a second elution was carried out (Eluate II).

RESULTS

Portions of a pooled rheumatoid serum (R-serum 1) and a pooled normal serum (N-serum 1) were fractionated according to method A. The agglutination titers for sensitized sheep cells and the protein concentrations of the sera and their fractions, diluted to original serum volumes, are presented in table 1.

From the data in table 1 it can be seen that the AAF was easily precipitable from the rheumatoid serum. Furthermore, the rheumatoid serum gave more precipitate at the first fractionation step than did the normal serum. This precipitate contained the bulk of the AAF activity.

In an attempt to pin down the AAF activity to known protein fractions, another pooled rheumatoid serum (R-serum 2) and a

TABLE 1

AAF ACTIVITY AND PROTEIN CONCENTRATIONS OF A RHEUMATOID SERUM, A NORMAL SERUM AND THEIR FRACTIONS, OBTAINED IN FOUR EXPERIMENTS ACCORDING TO FRACTIONATION METHOD A

	Sera not Absorbed Before Fractionation[1]			Sera Absorbed with Non-Sensitized Sheep Cells[1]			
	Exp. 1	Exp. 2		Exp. 3		Exp. 4	
	DAT	DAT	% prot.	SC	% prot.	SC	% prot.
R-serum	256	256	8.4	2048	8.4	4096	7.7
R-prec. fr. I	256	64	0.8	512	0.8	1024	0.8
R-prec. fr. II	32	8	3.9	32	4.0	8	3.8
R-snt. fr. II	0	0	4.4	0	3.2	0	2.9
N-serum		4	7.7	16	7.5	8	6.7
N-prec. fr. I		1	0.2	16	0.3	8	0.4
N-prec. fr. II		4	3.9	8	3.8	8	3.6
N-snt. fr. II		0	4.4	0	3.2	0	2.7

0 = No agglutination of sensitized nor nonsensitized sheep cells in dilution $\geq$ 1:4.

[1] When normal sheep cell agglutinins have been removed by absorption. the agglutination titer for sensitized sheep cells (SC) reflects the AAF activity. In nonabsorbed sera the differential agglutination titer (DAT) reflects the AFF activity.

TABLE 2

AAF ACTIVITY OF A RHEUMATOID SERUM, A NORMAL SERUM AND THEIR FRACTIONS, OBTAINED BY FRACTIONATION METHOD B

	Aggl. Titer for Sens. Sheep Cells
R-serum[1]	1024
R-fr. II	0
R-fr. III	512
R-fr. III-1	8
R-fr. III-2	256
R-fr. III-2-1	16
R-fr. III-2-2	256
R-fr. IV-V-VI	0
N-serum[1]	8
N-fr. III-2-1	16
N-fr. III-2-2	8

0 = No agglutination in dilution $\geq$ 1:4.

[1] Absorbed with nonsensitized sheep cells previous to fractionation.

pooled normal serum (N-serum 2) were subjected to fractionation according to method B. The titers of the sera and their various fractions are presented in table 2. The electrophoretic pattern of the AAF active fraction R-III-2-2 is shown in figure 1.

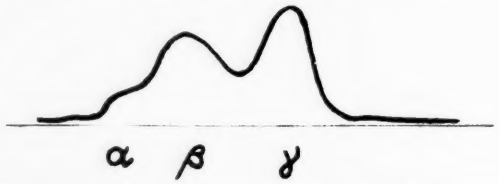

Fig. 1. — Electrophoretic pattern of the AAF-active R-fraction III-2-2. α = 8.5 per cent, β = 44.0 per cent, γ = 47.5 per cent.

The data presented in table 2 and figure 1 show that the AAF activity remained within the β-γ-globulin fraction (R-III, R-III-2 and R-III-2-2), whereas the fractions containing the immune gamma globulins (R-II) and the β-lipoproteins soluble at pH 6.3 (III-1) were practically devoid of AAF activity.

Because the AAF activity still was connected with three electrophoretically distinguishable globulin groups, the fractionation was continued according to method C. The titers and protein concentrations of a pooled rheumatoid serum (R-serum 3) and its fractions are presented in table 3. The electrophoretic patterns of fraction C and C-2 obtained by further purification of fraction C are shown in figures 2 and 3.

TABLE 3

AAF ACTIVITY AND PROTEIN CONCENTRATIONS OF A RHEUMATOID SERUM AND ITS FRACTIONS OBTAINED BY FRACTIONATION METHOD C

	Aggl. Titer for Sens. Sheep Cells	Per Cent Protein
R-serum[1]	2048	8.2
R-fr. C	256	0.6
R-fr. C-1	0	0.1
R-fr. C-2	512	0.5
R-fr. D	16	0.6
R-fr. F	0	0.8
R-fr. G	0	0.4

0 = No agglutination in dilution $\geq$ 1:4.

[1] Absorbed with nonsensitized sheep cells previous to fractionation.

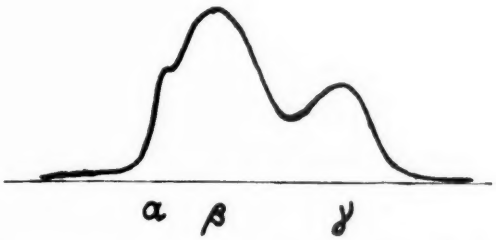

Fig 2. — Electrophoretic pattern of the AAF-active R-fraction C. $a = 10.6$ per cent, $\beta = 60.5$ per cent, $\gamma = 28.8$ per cent.

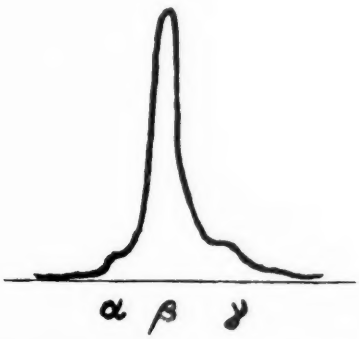

Fig. 3. — Electrophoretic pattern of the AAF-active R-fraction C-2. $a = 3.4$ per cent, $\beta = 90.0$ per cent, $\gamma = 6.6$ per cent.

The data presented in table 3 and figures 2 and 3 suggest that the AAF activity remains within the fraction which consists mainly of β-globulin (R-fr. C-2). On the basis of protein concentration, roughly a four-fold purification was achieved.

In addition to the fractionations described, attempts were made to purify the AAF by absorption elution procedures, using as adsorbents sensitized chicken cell »ghosts», *i.e.*, sensitized chicken cell stromata. By the use of chicken cell »ghosts» instead of whole chicken cells for virus purification, contamination with nonspecific intracellular protein material can be avoided to a great extent. Virus preparations of a high degree of purity have thus been obtained (6). For the same reason we chose chicken cell »ghosts» for our purpose.

In preliminary tests it was found that the action of AAF could be shown when whole chicken cells or chicken cell »ghosts» were used instead of sheep cells.

TABLE 4

AAF ACTIVITY AND PROTEIN CONCENTRATIONS IN ABSORPTION ELUTION TESTS WITH A RHEUMATOID SERUM AND A NORMAL SERUM

	Experiment 1			Experiment 2
	Titer for Sens. Sheep Cells	Per Cent of Protein		Titer for Sens. Sheep Cells
		Beckman	Kjeldahl[4]	
R-serum[1]	2048			2048
R-prec. fr. I[2]	1024	1.78	1.68	1024
—»— abs. w. sens. »ghosts» ..	32	0.63	0.66	256
R-eluate I[3]	64	0.09	0.03	128
—»— abs. w. nonsens. »ghosts»	64	0.29		
R-eluate II[3]	16			16
N-serum[1]	2			2
N-prec. fr. I[2]	4	1.09	1.03	2
—»— abs. w. sens. »ghosts» ..	0	0.84	0.80	0
N-eluate I[3]	0	0.06	0.03	4
—»— abs. w. nonsens. »ghosts»	0	0.45		
N-eluate II[3]	0			0

0 = No agglutination in dilution $\geq$ 1:2.

[1] Previous to fractionation, serum absorbed with nonsensitized sheep cells and nonsensitized chicken cell »ghosts».

[2] Obtained by fractionation method A. This fraction had been lyophilized and redissolved to original volume without any effect on its AAF activity.

[3] Sensitized »ghosts» used for absorption were eluated at +56°C for 5 minutes.

[4] Factor 6.25 was used to convert the nitrogen values to protein.

Table 4 shows the results obtained in the absorption elution experiments.

It will be seen from the data presented in table 4 that absorption with sensitized »ghosts» considerably decreased the AAF activity. In control experiments, not presented in the table, it was found that nonsensitized »ghosts» had no such effect upon the AAF activity. The data in table 4 also show that some of the AAF activity was recovered by elution at +56°C of the sensitized »ghosts» used for absorption. It should be mentioned that no AAF activity whatsoever was detectable in the three saline solutions used for washing of the sensitized »ghosts» before elution at +56°C.

On the basis of protein concentration, no significant purification of the AAF was achieved. Considering the possibility that at least some of the protein found in the eluates might be due to specific anti-»ghost» antibodies, released from the sensitized »ghosts» on elution at +56°C, the eluates were absorbed with nonsensitized »ghosts». The protein concentration, however, increased.

DISCUSSION

The writers suggest the following procedure for the preparation of the AAF active fraction, consisting mainly of β-globulin. The rheumatoid serum is brought to conditions: vol.1.2, ethanol 10 per cent, pH 6.3, ionic strenght 0.14, temp. —3°C. The precipitate formed is then brought to conditions: vol. 4.0, ethanol 20 per cent, pH 5.56, ionic strenght 0.05 acetate, glycine 0.55 M. The precipitate then formed is suspended in distilled water and lyophilized.

The ethanol fractionation experiments described indicate that the AAF is connected with a serum protein. Compared to other serum components, this protein is rather insoluble, resembling in that respect the »cold precipitable globulins» found by some authors to be increased in rheumatoid sera (7,11).

Attempts have been made to trace the AAF activity to an electrophoretically distinguishable protein group. The results, though strongly suggesting a connection of the AAF with β-globulin, do not eliminate, however, the possibility of a connection with a minor amount of γ-globulin.

The attempts to purify the AAF by absorption elution procedures with chicken cell »ghosts» were unsuccesful when estimation of purity was based on the protein concentration versus AAF activity. It remains to be studied whether »specific» techniques of this kind would provide a means for isolating the AAF from rheumatoid sera, free from other human serum constituents. Provided that the AAF possesses antigenic properties different from those of other antigens contained in human serum, it might prove feasible to produce specific anti-AAF sera by immunizing animals with AAF active eluates. A study along these lines is in progress.

SUMMARY

Fractionation experiments have been carried out by ethanol fractionation techniques in order to purify the agglutination activating factor (AAF) responsible for the agglutination of sensitized erythrocytes. A method of concentrating from rheumatoid sera a protein fraction consisting mainly of β-globulin and containing the AAF activity, is presented.

Furthermore, attempts to purify the AAF by absorption elution procedures using chicken cell »ghosts» are described.

REFERENCES

1. Ball, J.: Lancet 1950:*II*:520.
2. Brown, R., Bunim, J. J., and McEwen, C.: Ann. Rheumat. Dis. 1949:*8*:299.
3. Cohn, E. J., Strong, L. E., *et al.*: J. Am. Chem. Soc. 1946:*68*:459.
4. Deutsch, H. F., Alberty, R. A., *et al.*: J. Immunol. 1947:*56*:183.
5. Dounce, A. L., and Lan, T. H.: Science 1943:*97*:584.
6. Elford, W. J., Chu, C. M., *et al.*: Brit. J. Exper. Path. 1948:*29*:590.
7. Fritze, E., and v. Zezschwitz, F.: Z. Rheumaforsch. 1949:*8*:235.
8. Jawitz, E., and Hook, E. V.: Proc. Soc. Exper. Biol. & Med. 1949: *70*:650.
9. Miller, J. E., Lynch, E. R., and Landsbury, J.: J. Lab. & Clin. Med. 1949:*34*:1216.
10. Rose, H. M., Ragan, C., *et al.*: Proc. Soc. Exper. Biol. & Med. 1948: *68*:1.
11. Schwartz, T. B., and Jager, B. V.: Cancer 1949:*2*:319.
12. Waaler, E.: Acta Path. et Microbiol. Scandinav. 1940:*17*:172.
13. Wager, O.: Ann. Med. Exper. et Biol. Fenniae 1950:*28*:Suppl. 8.
14. Winblad, S.: Svenska läk. tidn. 1949:*26*:1.
15. Winblad, S.: Acta Med. Scandinav. 1952:*142*:450.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SENSITIVITY OF MICROCOCCI TO TEN ANTIBIOTICS

by

M. E. PARMALA

(Received for publication July 31, 1953)

The commonly-used antibiotics, especially penicillin, are continuously losing effectivity against micrococcic (staphylococcic) infections (1, 5, 7). Resistant strains, especially those of *Micrococcus pyogenes v. aureus* (coagulase positive), may also cause severe secondary infections during an antibiotic treatment (2). There is accordingly considerable interest in the sensitivity of micrococci to all the available antibiotics.

We have studied the activity of ten antibiotics to two known laboratory strains, Oxford and Orion, and 8 recently isolated strains (4 coagulase positive and 4 coagulase negative) with different sensitivities.

The sensitivity test was performed by the serial dilution method in nutrient broth. Most of the antibiotics were available in the Pfizer antibiotic diagnostic kit. They and the crystalline neomycin were directly dissolved in nutrient broth. The stock solution of erythromycin tablets (Erythrosin, Abbot) was made according to Dr. J. Sylvester (6), as follows: One 100 mg tablet is ground in a sterile mortar with 10—15 cc of acetone. The acetone solution is added to 500 cc of sterile distilled water. One cc of this solution is added to 9 cc of broth medium, giving a solution containing 20 mcg/cc of erythromycin.

The results are given in table 1.

TABLE 1

INHIBITORY CONCENTRATIONS OF THE ANTIBIOTICS FOR THE MICROCOCCUS STRAINS

Strain	Penicillin	Streptomycin	Aureomycin	Chloromycetin	Terramycin	Bacitracin	Polymyxin	Neomycin	Carbomycin	Erythromycin
Oxford	<0.39	<0.39	6.25 (3.12)	3.125	<0.39	2.5 (1.25)	25	<0.19	<0.39	0.078 (0.039)
Orion	<0.39	<0.39	6.25 (3.12)	3.125 (1.56)	<0.39	2.5	12.5	<0.19	<0.39	0.039
1340	6.25	50 (25)	100	3.125 (1.56)	>100	0.625	25 (12.5)	<0.19	<0.39	0.078 (0.039)
2287	<0.39	6.25	25. (12.5)	25	50 (12.5)	2.5	25	<0.19	<0.39	0.019
903	<0.39	100	100 (50)	100	100	2.5 (1.25)	12.5	<0.19	<0.39	0.019 (0.009)
2629	0.78 (0.39)	<0.39	3.125 (1.56)	3.125	<0.39	2.5 (1.25)	25 (12.5)	<0.19	<0.39	0.078 (0.039)
3110	0.78	<0.39	1.56 (0.78)	6.25 (3.12)	0.78	2.5	12.5	<0.19	<0.39	0.019
3114	12.5	0.78 (0.39)	3.125 (1.56)	3.125	<0.39	1.25	25	<0.19	<0.39	0.078
3083	50	3.125	3.125 (1.56)	3.125	<0.39	1.25	25 (12.5)	0.012	0.19 (0.09)	0.039
3172	<0.39	0.78 (0.39)	3.125 (0.78)	3.125 (1.56)	<0.39	0.625	25 (12.5)	0.012	0.39 (0.19)	0.019

Concentration for incomplete inhibition given in parentheses.
Penicillin and bacitracin units/cc, others mcg/cc.

DISCUSSION

According to table 1, even the most resistant strains 1340, 2287 and 903 can be »treated» with some of the usual antibiotics. Common for all the tested stains was their great sensitivity to carbomycin, erythromycin and neomycin. According to the literature (2, 7) the sensitivity of micrococci to carbomycin varies from 0.78 mcg/cc to 5.25 mcg/cc. The sensitivity of all our strains was as much as 2 to 4 times greater than the weakest concentration reported. For erythromycin inhibition concentrations from 0.01 mcg/cc to 1.6 mcg/cc have been given (2, 4), and most of our strains were sensitive even to the weakest concentration. According to Finland et al (3) the sensitive strains are usually 4 to 16 times more sensitive to erythromycin than to carbomycin, measured by the weight. We were able

to verify this. The activity of neomycin usually varies according to the literature (2) between 0.19 mcg/cc and 1.56 mcg/cc. The strains tested proved to be more sensitive. The growth of all strains was inhibited by 0.19 mcg/cc (= 0.04 units/cc) and that of the farther tested strains even by 0.012 mcg/cc (= 0.0025 units/cc). The dissimilarity may be due to the difference in the percentage of active factor in the unit of weight, which in our sample was 206 units/mg of neomycin.

It is to be remarked that strain 903, which generally was very resistant to several antibiotics, was especially sensitive to erythromycin.

SUMMARY

Ten micrococci strains were tested against ten antibiotics. Two of the strains were resistant to penicillin, 3 to streptomycin, 3 to aureomycin, 2 to chloromycetin, 3 to terramycin and all 10 to polymyxin. All strains were moderatery, sensitive to bacitracin and highly sensitive to carbomycin, erythromycin and neomycin.

REFERENCES

1. Banks, H. S.: Lancet 1952:I:560.
2. Dearing, W. H., and Heilman, F. R.: Proc. Staff. Med. Mayo Clin. 1953:*28:*121.
3. Finland, M., Wilcox, C., Wright, S. S., and Purcell, E. M.: Proc. Soc. Exp. Biol. 1952:*81:*725.
4. Haight, T. H., and Finland, M.: Proc. Soc. Exp. Biol. 1952:*81:*175.
5. Parmala, M. E.: Ann. Med. Exp. Biol. Fenn. 1953, (in press).
6. Sylvester, J. C.: personal communication.
7. Tanner, F. W., English, A. R., Lees, T. M., and Routien, J. B.: Antib. Chemother. 1952:*2:*441.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

ANTIBIOTICS AND EXPERIMENTAL TOXOPLASMOSIS

POLYMYXIN B-SULFATE, BACITRACIN, TERRAMYCIN, AUREOMYCIN AND SULFA IN EXPERIMENTAL TOXOPLASMOSIS [1]

by

PAUL GRÖNROOS [2]

(Received for publication August 28, 1953)

Sulfa preparations, specially sulfathiazole (6), sulfapyridine (6, 9) and sulfadiazine, have been proved to have a certain effect on experimental toxoplasmosis. As to the question which of the sulfa preparations is the best, and which of them are completely without effect, opinions are somewhat varying. In the experiment now reported, 6-sulanilamido-2.4-dimetyl-pyrimidine (Elkosin CIBA) was chosen as a control product.

Some investigators consider that aureomycin is without effect on Toxoplasma (7), but some laboratory experiments have shown that this is not quite true (4). A lowering of the dye-test titer in serum from people treated with aureomycin has also been observed (10).

Terramycin has been used in experimental toxoplasmosis in rabbits (5) and in white mice (3). Terramycin administered intramuscularly in doses of 1 mg/mouse was found to be completely ineffective. On the other hand aureomycin (0.4 mg per mouse)

The antibiotics have kindly been placed at my disposal by Chas. Pfizers et Co's principal agent in Helsinki the Havulinna OY.

[1] This investigation was aided by a grant from Svenska Vetenskapliga Centralrådet.

[2] With the technical assistance of Mrs. Pirkko Koivunen.

was only found to be effective when administered intramuscularly. Even large doses of aureomycin were ineffective when given orally (2).

Polymyxin and Bacitracin seem not to have been tried in toxoplasmosis.

Technic. — 10 days old embryonated hens' eggs were used in the experiments. The embryos were infected through the yolk sac with 0.2 ml of 1/10 dilution of peritonealexsudat from mice infected intraperitoneally four days previously. As a control, 0.2 ml of buffer solution (*p*H 7.4) was injected into a number of eggs intended as control eggs for the drugs. After 20 hours the drugs were injected in a non-recurrent dose of 0.2 ml into the yolk sacs of the infected and into the control eggs. The eggs were stored in an egg incubator at 36.5 C° and candled two times a day. From the eggs that died a sterility control was made on blood and Condradi-Drigalski agar.

Experiment. — To find out the toxic doses for embryonated eggs of the drugs in question, the drugs were injected into the yolk sacs in 0.2 ml of buffer solution containing 1/200 of the twenty-four hours' doses for a human being. The factor 1/200 was obtained by comparing the human twenty-four hour dose of sulfa with the known toxic dose of sulfa for embryonated hens' eggs, which is 20—40 mg in 0.2 ml. (1.)

Results in table 1.

TABLE 1

	No. of Eggs	Days after Inoculation					
		A			B		C
		2	3	5	12	13	
Sulfa 40 mg/0.2 ml	6	1		1	2	1	1
Aureomycin 10 mg/0.2 ..	6	1			3		2
Terramycin 10 mg/0.2 ..	6	1	2		1		2
Polymyxin 5 mg/0.2 ..	6		1	1	4		
Bacitracin 500 units/0.2	6	1		1	3		1
Buffer ph 7.4, 0.2 ml	6	1 (1)			3	2	

Column A contains the number of eggs that died so and so many days after inoculation.

Column B contains the number of eggs that hatched.

Column C contains the number of eggs in which the chickens had only picked small holes in the shells.

The parenthesis contains the number of eggs in which there was grow on the sterility controls.

On the basis of this experiment the following doses were used in the main experiment; sulfa 20 mg, aureomycin 5 mg, terramycin 5 mg, polymyxin B-sulfate 1 mg and bacitracin 250 units pro 0.2 ml buffersolution (*p*H 7.4).

Results in table 2.

TABLE 2

	No. of Eggs	A: Days after Inoculation											B		C
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Tox 1/1 0.02 ml	12		1		2	4	5								
Tox 1/10 in buffer	12				1	3 (1)	6	1	1						
» » and sulfa	13				1 (1)		1					1	6	1	3
» » and aureom. ..	13		1		1	2	3	2			2	2			
» » and terramycin	13		4				1	1	2		1	4			
» » and polymyxin	13					5	6	2							
» » and bacitracin	13		1(1)			3	3	5			1				
Buffer (ph 7.4)	14												9	2	3
» and sulfa.....	13							1					6	4	2
» and aureom.	13												11		2
» and terram.	13		1		1	2					1		4		4
» and polymyxin ..	13	1 (1)	1										5	4	2
» and bacitracin ..	13							1					9	2	1

Column A contains the number of eggs that died so and so many days after inoculation.

Column B contains the number of eggs that hatched.

Column C contains the number of eggs in which the chickens had only picked small holes in the shells.

The parentheses contain the number of eggs in which there was grow on the sterility controls.

Discussion. — From the table, it will be seen that terramycin also in the dose used was a little toxic. Sulfa, i.e., Elkosin (Ciba), had a very good effect, 10 of the 13 infected eggs survived the control. 7 of the sulfa-treated eggs hatched, and in 3 cases the chickens were only able to pick a hole in the shell. Parts of the brains and the livers of the 7 hatched chickens were inoculated intraperitoneally into mice, and Toxoplasma could be isolated from everyone of the seven chickens.

Aureomycin and terramycin had a weak effect, but bacitracin and polymyxin B-sulfat were without effect.

As it is probable that the drugs merely act on the extracellularly toxoplasmas, the effect would perhaps be more obevious if the time between infection and druginjection had been shorter.

SUMMARY

Sulfa (Elkosin, Ciba) has a good effect on experimental toxoplasmosis in embryonated hens' eggs. The chickens, however, remain Toxoplasma carriers. Aureomycin and terramycin have a weak effect, but bacitracin and polymyxin B-sulfate are without effect on toxoplasmosis.

REFERENCES

1. Adams, F. H., Cooney, M., Adams, J. M., and Kabler, P.: Proc. Soc. Exp. Biol. and Med. 1949:*70:*258.
2. Grassi, C.: Atti Soc. Lombarda Sci. med. biol. 1951:*7/1:*23.
3. Grassi, C., and Kåss, E.: Acta path. microbiol. Scand. 1952:*30/3-4:*304.
4. Kåss, E., and Steen, E.: Acta path. microbiol. Scand. 1951:*28:*165.
5. Puech, J.: Ann. Parasit. hum. comp. 1952:*27/4:*419.
6. Sabin, A. B., and Warren, J.: J. Bakt. 1941:*41:*8.
7. Summers, W.: Amer. J. Trop. M. 1949:*29:*889.
8. Warren, J., and Sabin, A. B.: Proc. Soc. Exp. Biol. and Med. 1942: *51:*15.
9. Weineman, D., and Berne, R.: J.A.M.A. 1944:*124:*6.
10. Westphal, A.: Zeitschr. f. Tropemned u. Parasit. 1950:*Band 1:*heft 4.
11. Wolfschlag, H. J.: Z. Kinherheilk. 1951:*69/6:*564.

FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF HELSINKI

METABOLISM OF ACETOIN AND DIACETYL IN LIVER TISSUE[1]

SOME OBSERVATIONS

by

J. JÄRNEFELT

(Received for publication September 3, 1953)

Acetoin (acetylmetylcarbinol) and diacetyl have long been known to be products of the metabolism of living organisms. They have been found in bacteria (1—5), in yeast (20—21) and in animal organisms (6—10). Recently Juni has treated the formation of acetoin in bacteria, yeast and mammalian tissue (11—12). The elimination of acetoin in the animal organism has been treated by Greenberg (15), while Green *et al.* have investigated the elimination of diacetyl in animal tissues (16) and Berl and Bueding the metabolism of acetoin in filariae (17).

In the following preliminary report the writer sets forth some observations on the metabolism of acetoin and diacetyl in liver tissue *in vitro*. It has became apparent that the metabolism of these compounds differs considerably in liver homogenates and in liver slices. The homogenates are incapable of destroying acetoin, while slices have a clear activity towards it. Also diacetyl behaves differently in homogenates and in slices.

METHODS

Rat livers prepared in two different ways were used throughout this investigation. The homogenates were prepared in a Waring

[1] This investigation has been supported by a generous grant from the Foundation for the Research of Alcohol Problems.

blendor with cold physiological NaCl as the suspending medium and diluted to contain 1 part of liver in 4 parts of homogenate. The liver slices were cut with a razor blade in cold physiological NaCl. In the experiments either 5 ml of the homogenate or 1 g of liver slices in 5 ml of physiological NaCl were used as sources of enzyme. The rest of the reaction mixture was in both cases of the same composition: 3 ml m/5 phosphate buffer, *p*H 7.4, and 2 ml (ad 10 ml) of substrates, activators and water. Acetoin and diacetyl were used in 0.1 % solutions (1 ml = 1 mg), Na-pyruvate and acetaldehyde in 1 % solutions (1 ml = 10 mg). Mn^{++}-ions were added as a 0.5 % $MnSO_4$ solution. The diphosphothiamine was prepared from thiamine according to Tauber (14). The constituents of the mixture were added in the following order: buffer, activators, pyruvate, liver preparation, substrates, acetaldehyde. Before addition of the substrates the flasks were aerated with tank oxygen to obtain aerobic conditions.

To enable the course of the enzymatic reaction to be followed 1 ml samples were pipetted from the mixture immediately after addition of the substrate and after 30, 60 and 120 minutes had elapsed. Sometimes only the first and last of these samples were taken. For the determination of acetoin and diacetyl in these samples Westerfeld's method (19) in a somewhat modified form was used. The samples were pipetted in 1 ml of 5 % metaphosphoric acid and allowed to stand some time for the precipitation of proteins, then diluted to 10 ml with water and centrifuged. The clear supernatant was distilled according to Westerfeld in vacuo, in the presence of NaCl, practically to dryness. A 5 ml aliquot of the distillate was used for the colorimetric determination. Making use of the fact that the colour develops much faster in the case of diacetyl (about 10 min.) than in that of acetoin (60 min.) it was possible to determine acetoin and diacetyl simultaneously by measuring the colour at 10 and 60 min. From measurements with pure acetoin and diacetyl solutions the following formulas were derived:

Take

E_{10} = the extinction at 10 min.
E_{60} = » » » 60 min.
E_x = » » produced by acetoin, and
E_y = » » » » diacetyl.

Then (the factors were calculated from empirical data)

$$E_x = 1.3 \cdot E_{50} - 1.17 \cdot E_{10}.$$
$$E_y = 1.3 \cdot E_{10} - 0.34 \cdot E_{60}.$$

From E_x and E_y the corresponding amounts of the substances are obtained in the usual manner from an extinction curve. The measurement of the extinctions was performed with a Beckman model B spectrophotometer at a wavelength of 5300 Å. In our experience the method described above is convenient and satisfactory. The accuracy is of course not very high, but seems to be sufficient.

RESULTS

The results are shown in Tables 1—4. In Table 1 the experiments relating to homogenates are collected. It will be seen that during an incubation time of 2 hours no diminution of acetoin took place, and that acetaldehyde or pyruvate had no effect thereon (exp. 1—4).

TABLE 1

HOMOGENATES

No.	Added, Theory				DPT 200 γ	$MnSO_4$ 5 mg	Found, γ			
	Acetaldehyde mg	Pyruvate mg	Acetoin γ	Diacetyl γ			Acetoin		Diacetyl	
							0^h	2^h	0^h	2^h
1	—	—	100	—	—	—	94	97	0.8	0.8
2	1	—	100	—	—	—	110	99	3.6	1.1
3	—	1	100	—	—	—	89	85	1.6	0.8
4	1	—	—	—	—	—	0	7	0	0
5	—	—	—	100	+	+	22	28	62	31
6	1	—	—	100	+	+	28	57	55	32
7	—	1	—	100	+	+	21	36	76	46
8	1	—	—	—	+	+	0	21	0	0

*p*H 7.4, incubated 2^h in a water bath at 37 C°.

Diacetyl, however, was clearly active as substrate in homogenates fortified with diphosphothiamine and Mn^{++} (exp 5—8). Pyruvate and acetaldehyde seemed to have some effect on the disappearance of diacetyl and the formation of acetoin from it. The ratio between the acetoin formed and the diacetyl which has disappeared varies in the experiments 5—7:

5) $\frac{+\text{acetoin}}{-\text{diacetyl}} \sim 0.2$, 6) $\frac{+\text{acetoin}}{-\text{diacetyl}} \sim 0.33$, 7) $\frac{+\text{acetoin}}{-\text{diacetyl}} \sim 0.5$

In exp. 6 the acetoin formed by acetaldehyde alone (21γ) is subtracted. Pyruvate alone did not give any increase of acetoin. It was necessary to use homogenates fortified with diphosphothiamine and Mn^{++}, as the results in pure homogenates were inconclusive. The high 0^h-acetoin values in exp. 5—7 are apparently due to impurities in the diacetyl. After distillation of the diacetyl at 88 C° these impurities disappeared as is shown in Table 2, exp. 4—6.

TABLE 2

SLICES

Exp.	Added, Theory		Found							
	Acetoin γ	Diacetyl γ	Acetoin, γ				Diacetyl, γ			
			0^m	30^m	60^m	120^m	0^m	30^m	60^m	120^m
1	100	—	86	78	60	46	1.2	4.8	0	3.6
2	100	—	95	71	70	54	5.2	3.8	0	8.0
3	100	—	86	85	66	52	3.4	4.0	0	5.3
4	—	100	1.4	57	59	59	66	26	13.9	13.1
5	—	100	0.5	59	76	85	77	29	14.7	10.0
6	—	100	5.7	54	66	61	114	25	7.2	9.2

pH 7.4, incubated 30—120^m in a water bath at 37 C.

In Table 2 the corresponding experiments with liver slices are given. One can see that the amount of acetoin has clearly decreased, but that no diacetyl has been formed. (exp. 1—3). In exp. 4—6 a very heavy decrease of diacetyl is seen, which is followed by the formation of an aequimolar amount of acetoin. Contrary to the homogenates the ratio

$$\frac{+\text{acetoin}}{-\text{diacetyl}} \sim 1$$

(The 0^m-value for diacetyl in exp. 6 is clearly too high, probably due to an experimental error).

In Table 3 the dependence of acetoin disappearance on the initial concentration is shown. It is a curious fact that no disappearance is seen at high initial concentrations (exp. 3—4) while at lower concentrations the phenomenon is clear. Table 4 on the other hand, shows the same experimental series for diacetyl. The reaction

TABLE 3

SLICES

No.	Acetoin Added, Theory γ	Found			
		Acetoin, γ		Diacetyl, γ	
		0^h	2^h	0^h	2^h
1	50	38	34	0.8	2.4
2	200	158	96	8.4	7.4
3	600	455	455	63	21
4	1 000	685	680	40	40

Conditions same as in Table 2.

TABLE 4

SLICES

No.	Diacetyl Added, Theory γ	Found				$\frac{+\text{Acetoin}}{-\text{Diacetyl}}$
		Acetoin, γ		Diacetyl, γ		
		0^h	2^h	0^h	2^h	
1	50	0	26	26	2.9	1.1
2	200	0	129	112	13.1	1.3
3	400	0	190	225	56	1.1
4	600	0	306	345	101	1.2

Conditions same as in Table 2.

velocity increases with increasing initial concentration. The ratio between acetoin formed and diacetyl consumed is given in the last column, the values are essentially the same as those derived from Table 2.

DISCUSSION

The abovementioned results are not so easy to explain. The lack of activity towards acetoin in the homogenates is probably due to some dilution effect. Another possibility would of course be the destruction of some component of the enzymatic system during the homogenization. As to the mechanism of acetoin disappearance in liver slices it seems fairly clear that acetoin is not oxidized to diacetyl. When acetoin is consumed no increase in diacetyl concentration is observed. Such a situation could be possible, of course, if the diacetyl formed were metabolized with a velocity greater than the velocity of formation. This is actually the case, but during the metabolism of diacetyl an aequimolar

(at least) amount of acetoin is invariably formed. This would lead to the paradox that no decrease in acetoin concentration ought to be detected. The mechanism of the metabolism of acetoin is still obscure. In this connexion however, the author wishes to express the following thoughts. A possible line for the metabolism of acetoin could be a splitting of the molecule into two parts, through some mechanism, probably involving coenzyme A, being used for acetylation purposes. The fact that Doisy and Westerfeld have found that acetoin increased the acetylation of p-aminobenzoic acid (18) and that Kinnunen arrived to the same result in the case of sulfonamides (22), can be taken in favour of the hypothesis proposed above.

The fate of diacetyl in metabolism, seems, at least partly, to be a conversion into acetoin. In liver slices this conversion is total. Green *et al.* have described an enzyme, which they call diacetyl mutase (16), and which catalyses the dismutation of 2 moles diacetyl into 1 mole acetoin and 2 moles acetic acid. The quantitative results in our homogenates fit into this system, to some extent at least, but the activity of the slices cannot be explained by it. In this respect the author's experiments could better be explained through some sort of transcarboligation, as described by Akabori and Shimazu (13). In any case it seems clear, that the metabolic pathway of diacetyl primarily goes through acetoin.

Work for a thorough explanation of these problems is in progress.

SUMMARY

1) A method based on Westerfeld's (19) is given for the simultaneous determination of acetoin and diacetyl in the same sample.

2) It is found, that rat liver homogenates do not use acetoin as substrate, while liver slices do this.

3) In liver homogenates diacetyl is partially converted into acetoin.

4) In liver slices however, all diacetyl which has disappeared, is recovered as acetoin.

5) All experiments in this study were carried out under aerobic conditions.

6) Some questions, raised by the results above, are discussed.

REFERENCES

1. Voges, O., and Proskauer, B.: Z. Hyg. u. Infekt. Kr. 1898:*28:*20.
2. Harden, A.: Proc. Roy. Soc. B 1906:*77:*424.
3. Harden, A., and Norris, D.: J. Physiol. 1911:*42:*332.
4. Harden, A., and Norris, D.: Proc. Roy. Soc. B 1911:*77:*399.
5. Schmalfuss, H., and Barthmeyer, H.: Ztschr. physiol. Chem. 1928: *176:*282.
6. Neuberg, C., and Gottschalk, A.: Biochem. Ztschr. 1925:*162:*484.
7. Schmalfuss, H.: Ztschr. physiol. Chem. 1930:*188:*279.
8. Schmalfuss, H., and Schaake, H.: Ibid. 1931:*200:*169.
9. Schmalfuss, H.,: Ibid. 1934:*227:*247.
10. Gorr, G.: Biochem. Ztschr. 1932:*254:*12.
11. Juni, E.: J. Biol. Chem. 1951:*195:*715.
12. Juni, E.: Ibid. 1951:*195:*727.
13. Akabori, S., and Shimazu, Y.: Proc. Japan Academy 1952:*28:*36.
14. Tauber, H.: J. Biol. Chem. 1938:*125:*191.
15. Greenberg, L. A.: Ibid. 1943:*147:*11.
16. Green, D. E., Stumpf, P. K., and Zarudnaya, K.: Ibid. 1947:*167:*811.
17. Berl, S., and Bueding, E.: Ibid. 1951:*191:*401.
18. Doisy, E. A., and Westerfeld, W. W.: Ibid. 1943:*149:*229.
19. Westerfeld, W. W.: Ibid. 1945:*161:*495.
20. Neuberg, C., and Hirsch, J.: Biochem. Ztschr. 1921:*115:*282.
21. Neuberg, C., and Simon, E.: Ibid. 1925:*156:*374.
22. Kinnunen, O.: Acta Physiol. Scand. 1946:*13:*suppl. 44.

FROM THE FIRST AND SECOND WOMEN'S CLINICS AND THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

DETERMINATION OF BACTERIAL SENSITIVITY BY THE QUICK-TEST AND DISC METHODS

COMPARATIVE STUDIES

by

O. WIDHOLM and M. E. PARMALA

(Received for publication September 4, 1953)

The high incidence of pyelitis and other diseases of inflammatory origin in the patients of the Women's Clinic, Helsinki, has shown the necessity of a quick and simple determination of bacterial resistance. For that purpose it was decided to adopt the sensitivity determination tablets manufactured by Roskilde Co. for clinical use.

It was necessary before starting to use them to compare this method with sensitivity determinations of the same samples by the routine disc method employed by the University Department of Serology and Bacteriology.

MATERIAL AND METHODS

The investigation series consisted of 50 samples of sterile urine, in which bacteria had been found microscopically. A quick resistance determination was made of the samples at the Women's Clinic laboratory and simultaneously identical samples were submitted to the Department of Serology and Bacteriology for bacteriological examination and sensitivity determination.

On the basis of the bacteriological examination the series was divided into the bacterial groups given in Table 1.

TABLE 1

CLASSIFICATION OF THE BACTERIAL STRAINS IN OUR SERIES

E. coli	30	Klebsiella	4
Proteus	2	Aerogenes	1
Alkalesc. Dispar	1	Paracoli	1
Microc. pyog. v. aureus	3	Microc. saprof.	3
Microc. pyog. and aerogenes	1	Microc. pyog. and enteroc.	1
E. coli and microc. pyog.	1	E. coli and providence	1
E. coli and faecalis alkalig.	1		

The blood agar plates required for the determinations at the Women's Clinic were produced by Messrs. Orion, drug manufacturers from whom fresh plates were ordered as required.

Since the Women's Clinic has restricted facilities for bacteriological examination, the dilution of urine sediment with saline was carried out according to the microscopical examination of the sediment (Gram stain).

TABLE 2

THE DILUTION OF URINE SEDIMENTS FOR QUICK-TEST SENSITIVITY DETERMINATION

Bacteria per field in microscope	Dilution for the inocul.
over 30 bacteria in the field of vision	1:10,000
5—30 » » » » » »	1: 5,000
1— 5 » » » » » »	undiluted
no bacteria on staining	undiluted

0.2 ml of diluted bacterial suspension was subsequently inoculated into one plate. After inoculation the sensitivity tablets were placed on the plates (6 tablets per plate) and the plates were kept in an incubator at +37°C. Readings were taken after 18—20 hours. Evaluation was based on the diameter of the inhibition zone:

0 = 0 cm
1 = approx. 0—1 cm
2 = 1—2 cm
3 = over 2 cm

The sulfathiazole and antibiotic content of the tablets used is given in Table 3.

TABLE 3

THE DRUG CONCENTRATIONS OF THE TABLETS AND DISCS AND THE CONCENTRATION RATIO.

	Tablets	Discs	Tablets/Discs
Sulfathiazole	2 mg	0.1 mg	*20*
Penicillin	50 units	10 units	*5*
Streptomycin	5 mg	0.02 mg	*250*
Aureomycin	5 »	0.05 »	100
Chloromycetin	5 »	0.025 »	200
Terramycin	5 »	0.025 »	200

Sensitivity determinations at the University Department for Serology and Bacteriology were effected by the disc method (1), the only difference being that the antibiotic concentrations were those indicated in Table 3 and the bacterial dilution of the 4-hour serum broth cultures was as follows: streptococci 1:500, micrococci 1:5,000 and gram-negative rods 1:50,000. All the determinations were made in the same plate, diameter 15 cm.

CONTROL DETERMINATIONS

In order to ascertain the error arising for technical reasons we effected simultaneous determinations of the sensitivity to the 5 antibiotics and sulfathiazole with 2 E. coli and 2 Micrococcus pyog. v. aureus strains on 10 different days by each of the two methods. The strains submitted for quick-determination were suspended in serum-broth in dilutions equivalent to 50 bacteria per field of vision in native staining. If a deviation of ± 1.5 cm in the reading is accepted as a technical error, the mean values for the quick-test are exceeded only once or twice in all the determinations (240) together, approx. 0.5 per cent. The corresponding error for the disc method occurred approx. 15 times more frequently (with sulfathiazole 2/40, penicillin 1/40, streptomycin 3/40, aureomycin 6/40, chloromycetin 6/40 and terramycin 3/40 errors.) The rather unexpectedly high percentage of diverging results with the disc method is probably due to the removal of discs after 15 to 20 min. before the plates were inoculated.

When comparing the results of the two tests the discrepancies were: Micrococci, sensitive to penicillin by disc method, proved to

be resistant 18 times in 20 determinations by the quick-test. This difference is probably due to the relatively higher penicillin concentration employed in the disc method. With E. coli strains the greatest differences emerged with streptomycin and chloromycetin, both of which gave slightly »more sensitive» results with the quick-test.

CLINICAL TESTS

Table 4 gives the results with the 30 E coli strains and the other 20 single or mixed bacterial strains (see Table 1) isolated from urine and tested by both methods.

TABLE 4

RESULTS OF SENSITIVITY TESTS OF THE CLINICAL SAMPLES

	Sulfath.	Penicill.	Strept.	Aureom.	Chlorom.	Terramyc.
E. coli						
Concordant results	19/30	29/30	14/30	24/30	26/30	28/30
Discordant results						
Quick-test more sensitive	10/30	1/30	16/30	2/30	4/30	0/30
Disc-test more sensitive	1/30	0/30	0/30	4/30	0/30	2/30
Other bacteria						
Concordant results	13/20	17/20	15/20	18/20	14/20	19/20
Discordant results						
Quick-test more sensitive	3/20	2/20	5/20	1/20	6/20	0/20
Disc-test more sensitive	4/20	1/20	0/20	1/20	0/20	1/20

We can see from the figures that the determinations with aureomycin and terramycin give consistent values. The previously mentioned difference with penicillin cannot be shown in this material as it included but few strains sensitive to penicillin. With sulfathiazole, streptomycin and to some extent also with chloromycetin, a larger proportion of »sensitive» results are found by the quick-test.

As can be seen from Table 3, the streptomycin content is relatively highest in the tablets, and so we get the strains sensitive to

streptomycin with tablets. The most astonishing finding is the sensitivity of strains towards sulfathiazole with the quick-test in spite of the relatively low sulfathiazole content of the tablets. This might be due to the slow diffusion of sulfathiazole into the plates apparently imcomplete when the discs are removed.

THERAPEUTICAL RESULTS

The patients were treated according to the sensitivity determinations made at the Women's Clinic. Instead of sulfathiazole Metizol mite tablets, 5 × 2 were used. The dosage of antibiotics has followed the general usage. Where possible, the therapeutical results have been checked up.

In 20 out of 50 cases the therapeutical result was concordant with both methods. In 7 cases the results have not been checked. In 4 cases the therapy was not succesful. The cases revealing discordance between the two sensitivity determinations are given in Table 5.

TABLE 5

SUCCESFUL THERAPEUTICAL RESULTS CONTRARY TO THE RESULT OBTAINED BY THE OTHER DETERMINATION

	Treatment in agreement with the quick-test result, but not with the disc-test			Treatment in agreement with the disc-test result, but not with the quick-test		
	E. coli	Others	Total	E. coli	Others	Total
Sulfathiazole	8	2	*10*	1	1	2
Penicillin	0	1	1	0	1	1
Streptomycin	2	2	*4*	0	0	0
Chloromycetin	0	1	1	0	0	0
Total	10	6	16	1	2	3

Table 5 shows that the differences are seen mainly in the sulfathiazole and streptomycin groups, in accordance with the fact that sulfa drugs and streptomycin gave more sensitive results with the quick-test and are the drugs more generally used for the treatment of urinary infections.

CONCLUSIONS

The technical error with the quick-test remains small compared with that in the disc method when the discs are removed.

The clinical material shows that, with 50 urine samples, the quick-test gives, with sulfathiazole approx. 25 per cent more sensitive results, with streptomycin approx. 35 per cent more sensitive results and with chloromycetin approx. 20 per cent more sensitive results. With the other antibiotics the results are practically identical (with gram negative rods).

According to this and to the therapeutical results the quick-test in use at the First and Second Women's Clinics has proved itself a quick and reliable method of determining sensitivity in infections of the urinary tract.

REFERENCE

1. KAIPAINEN, W. J.: Ann. Med. Exp. Biol. Fenn. 1953:*31*:149.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, UNIVERSITY OF HELSINKI

STUDIES ON THE NUCLEIC ACIDS OF THE OUTER ORBITAL GLAND OF THE WHITE RAT

by

T. KUUSI and H. TEIR

(Received for publication September 15, 1953)

In previous experiments (14) it was established that experimentally induced degeneration in the orbital gland of the white rat stimulates mitosis in homologous organs. A similar stimulation was also obtained by parenteral injections of orbital gland tissue suspensions. Little is known about the chemical nature of the mitotic stimulator active in these experiments. Some facts indicate, however, that nucleic acids may be involved. We know, for instance, that the activity of the suspension diminishes with increasing age of the donator rat (15); likewise the ribonucleic acid (RNA) content of the organs diminishes with increasing age (3). Further, the mitotic stimulator appears to be thermostable (15); therefore the proteins which otherwise might be thought to play a role here as tissue-specific agents, are excluded. Because of their complex character and great variability the nucleic acids would be structurally suitable as tissue-specific agents. Chargaff (5) considers it probable that desoxyribonucleic acid (DNA) is responsible for the species specificity, RNA being an organ-specific substance; RNA preparations isolated from the homologous organs of different animal species resemble one another more than RNA isolated from different organs of the same animal. It should also be mentioned that fractions containing RNA or DNA act as growth

An investigation aided by a grant from Alfr. Kordelin's Foundation and President J. K. Paasikivi's Fund for Cancer Research.

stimulators elsewhere, e.g. in bacteria (7), and in the chorio-allantoic membrane of the hen's egg (12). Marshak and Walker's (9) investigations should further be mentioned; these workers showed that chromatin fractions brought about mitotic stimulation in regenerating liver. We therefore considered it important, when studying the chemical nature of the mitotic stimulator in question, to pay particular attention to the occurrence of nucleic acids in the orbital gland and in suspensions prepared from it.

CYTOLOGICAL STUDY OF NUCLEIC ACID CONTENT

In order to obtain a general idea of the nucleic acid content of the orbital gland, the basophilia of the orbital gland of rats of different ages (1, 2 and 3 weeks and 5 months) was studied, using Unna-Pappenheim's methyl green-pyronine staining method as modified by Brachet (4). At the same time, for comparison, we stained liver, skin, stomach, and parotid and Harder's gland of the same rats. To make sure that the basophilia was caused by RNA, the preparations were treated with ribonuclease (Worthington's crystalline preparation) according to Brachet's method (2, 4).

The investigation proved that the orbital gland tissue was markedly basophilic and the cytoplasmic basophilia was clearly more pronounced than in the liver, which was used for comparison; the liver is considered to be particularly rich in RNA (6).

When investigating the occurrence of cytoplasmic basophilia in orbital glands of different ages, we found that at the age of 1 and 2 weeks the cytoplasm was throughout basophilic and cells were cuboidal. At later stages the cells had become high pyramidal and basophilia occurred in the periphery of the acini around the nucleus in the basal portion of the cell, the apex of the cell being only slightly basophilic. Since at an early stage the entire cytoplasm was basophilic, while at later stages basophilia was found only in the basal portion of the cell, it seems as if the total basophilia decreases with increasing age. The basophilia was not clearly localized in granular or mitochondrial structures but evenly distributed over the entire basal cytoplasm. From those orbital gland preparations which had been treated with ribonuclease the cytoplasmic basophilia had entirely disappeared. Thus the source of the basophilia in the orbital gland is ribonucleic acid.

In the organs studied for comparison we found that with regard to the basophilia the parotid gland tissue was of about the same order as the orbital gland. In Harder's gland, on the contrary, the basophilia was of a lesser degree. The basophilia of the liver tissue, too, was slighter than that of the orbital tissue and moreover of a different type, being localized, from the age of about 2 weeks, to the mitochondria, which were scattered in the cytoplasm. In the basal layer of the mucosa of the stomach the chief cells were highly basophilic, even more so than the cells in the orbital gland (3).

CHEMICAL DETERMINATION OF NUCLEIC ACIDS

Since the above cytological study gave us reason to believe that the orbital gland tissue has a high ribonucleic acid content, we thought it useful to confirm the result obtained by means of chemical determination. Thus we also obtained an idea of the quantity of desoxyribonucleic acid and mononucleotides in the orbital tissue. As basic material we chose orbital gland tissue from 2-week-old rats, since in previous experiments on mitotic stimulation this material proved most effective and has generally been used for the preparation of active suspensions (15). The determination was made by perchloric acid extraction and measurement of the UV-absorption of the extracts (10). The mononucleotides, the RNA and the DNA, were determined separately, the quantities being calculated on the basis of Beckman absorption values and dilutions of the extracts.

The absorption curves of the extracts are depicted in fig. 1. The quantities of the different nucleic acid fractions are as follows:

	Fresh weight per cent	Dry weight per cent
Mononucleotides	0.07	0.3
RNA	0.89	3.9
DNA	0.68	3.0

The result shows that the quantity of mononucleotides in the orbital gland tissue is comparatively small. Thus a fresh orbital gland tissue suspension probably contains but a small quantity of nucleic acid in a water-soluble or »free» form, the bulk occurring as ribo- or desoxyribonucleic acid. The quantity of RNA is considerable (cf. Davidson, 1950) as was to be expected on the basis of the cytological study.

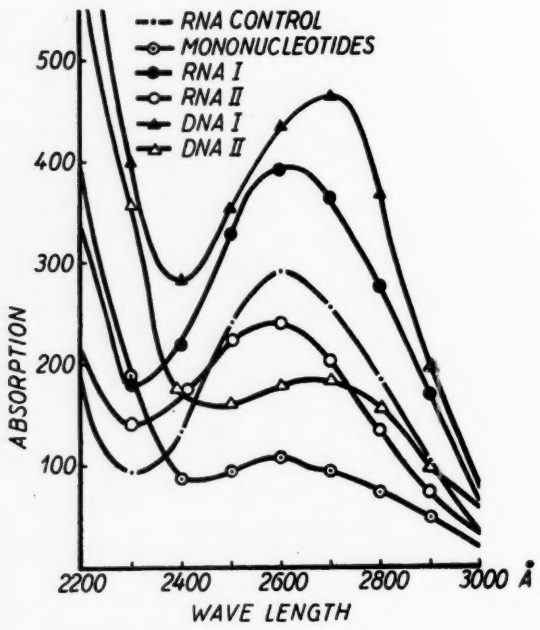

Fig. 1. — Ultraviolet absorption curves of the nucleic acid fractions.

RNA control: 20 γ/1 ml.
Mononucleotides: dilution 1: 1.
RNA I: dilution 1: 4.
RNA II: dilution 1: 4.
DNA I: dilution 1: 4.
DNA II: dilution 1: 2.

INFLUENCE OF HEATING ON THE NUCLEIC ACIDS

Heating rather increases than reduces the mitosis-stimulating effect of the orbital gland suspension (15). In particular it seems as if the heated suspension has a wider range of activity than an unheated suspension. The most suitable method of heating proved to be 15 minutes at 100° C. It now appeared of interest to know how such heating would influence the nucleic acids in the orbital gland tissue. It was to be expected that at least the RNA, which is well-known to be labile, would to a large extent become hydrolyzed. This problem was first investigated by cytological methods using Unna-Pappenheim's methyl green-pyronine staining.

When comparing the staining of the unheated control preparation and of orbital gland tissue heated for 15 minutes at 100° C, we observed that the cytoplasmic basophilia of the latter had almost entirely disappeared. Since for technical reasons the staining

properties of a whole orbital gland and not of an orbital gland suspension were investigated, it is obvious that the extraction of the RNA from mashed orbital gland tissue must be still greater. Thus the RNA in the heated orbital gland tissue has been almost totally released from the tissue structure and has become water-soluble. As regards the DNA, it also seems to have changed in one way or another, since in the staining of the nuclei a qualitative change was observed: in the unheated tissue the nuclei normally stained bluish green, while in the heated tissue they appeared red. Thus the DNA of the heated tissue stained with pyronine and not with methyl green. The importance of this change in staining properties is not fully understood and very varying explanations for it have been put forward (1, 8, 11, 13, 16). It should also be mentioned that the Feulgen reaction in a heated preparation was slightly weaker than in an unheated preparation.

Since the result of this cytological study was somewhat ambiguous, a complementary investigation was performed by means of chemical methods. In this investigation the above mentioned method of Ogur and Rosen was used in order to compare nucleic acid fractions with the corresponding fractions in unheated tissue. True, there will be a methodical error insofar as this method has not been applied to a tissue heated in advance, but in any case an idea is obtained of the change in the solubility of the nucleic acids as a result of heating.

As in the previous experiment, orbital gland tissue of 2-week-old rats was used. By means of Ogur-Rosen's method the mononucleotides (fraction soluble in cold 1 per cent $HClO_4$), the RNA (fraction soluble in cold 10 per cent $HClO_4$) and DNA (fraction soluble in hot 10 per cent $HClO_4$) were determined in heated suspensions and the quantities calculated on the basis of Beckman absorption values and dilutions of extracts.

The result is best seen from a comparison between the values for the different nucleic acid fractions in unheated and heated suspensions:

	Unheated	Heated	Difference
Mononucleotides	4.4%	14.7%	+10.3
RNA	54.3%	57.6%	+ 3.3
DNA	41.3%	27.7%	—13.6

This demonstrates that in a heated suspension the most readily soluble fraction is clearly increased and the least soluble one clearly reduced. The nature of the fractions was not more closely identified

and it is therefore not quite certain whether they comprise exactly the same components as in the normal Ogur-Rosen procedure. Since the cytological picture indicates that heating principally reduces the RNA and only to a small degree the DNA, it seems probable that the heating of the suspension changes part of the DNA into a form in which it dissolves already in cold 10 per cent $HClO_4$ and that the increase in mononucleotides originates from the RNA. It would seem that the cytological and the chemical investigation yield diverging results insofar as the »RNA» fraction, which according to the chemical investigation rather increased than decreased, does not stain with pyronine after heating. The nature of this change in the staining is not clear and requires further investigation.

CONCLUSIONS

The investigation throws light upon the basophilia of the orbital gland and the change in this with increasing age; the decrease in the basophilia resembles the decrease in the mitotic stimulation with increasing age of the donator. Further the investigation shows that the cytoplasmic basophilia of the orbital gland is caused by the ribonucleic acid in the gland, and that the quantity of this is considerable according to chemical determination. It was also established that heating of the orbital gland suspension considerably increases the solubility of the nucleic acids, particularly that of the RNA. This result gives a good basis for further research, the object of which should be to elucidate whether the nucleic acids in the orbital gland tissue are responsible for mitotic stimulation. Since the RNA content is high, it is possible both to separate RNA from the orbital tissue and to test its effect on mitotic stimulation, and on the other hand, to eliminate the RNA from the orbital tissue and to find out whether such a RNA-free suspension is still active. Such an investigation would answer the question of whether the mitotic stimulator could possibly be identified with the tissue RNA. It should further be mentioned that the mode of binding of the RNA apparently does not influence the effect of the suspension to any marked degree since both an unheated and a heated suspension give positive results; in the former case the RNA is bound to the tissue proteins, whereas in the latter it occurs free in solution.

Acknowledgement: We are much indebted to Professor E. Uroma, M.D., Director of the State Serum Institute, who kindly allowed to use the Beckman spectrophotometer in his institute.

REFERENCES

1. Alfert, M.: Biol. Bull. 1952:*103:*145.
2. Brachet, J.: Enzymologia 1941:*10:*87.
3. Brachet, J.: Embryologic chimique. Paris-Liege 1944.
4. Brachet, J.: Quart. Journ. Micr. Sci. 1953:*94:*1.
5. Chargaff, E.: Experientia 1950:*6:*201.
6. Davidson, J. N.: The Biochemistry of the Nucleic Acids. London-New York 1950.
7. v. Euler, H., and Jaarma, M.: Arkiv f. Kemi, Min. och Geol. 1946: *23 A:* 1.
8. Kurnick, N. B.: Exper. Cell Res. 1950:*1:*151.
9. Marshak, A., and Walker, A.: Am. Journ. Physiol. 1945:*143:*226.
10. Ogur, M., and Rosen, G.: Arch. Biochem. 1950:*25:*262.
11. Pollister, A. W., and Leuchtenberger, C.: Proc. Nat. Acad. Sci. 1949:*35:*111.
12. Shaw, E. I., and Shaver, J. R.: Experientia 1953:*9:*140.
13. Taft, E. B.: Exper. Cell Res. 1951:*2:*312.
14. Teir, H.: Soc. Scient. Fenn., Comm. Biol. 1951:*13:*1.
15. Teir, H.: Growth 1952:*16:*85.
16. Vercauteren, R.: Enzymologia 1950:*14:*134.

FROM THE DEPARTMENT OF MEDICAL CHEMISTRY, UNIVERSITY OF HELSINKI

THE ROLE OF VITAMIN C IN THE MUCOPOLYSACCHARIDE METABOLISM OF THE SKIN

STUDIES ON FREE MUCOPOLYSACCHARIDES AND MAST CELLS IN THE INTACT SKIN AND DURING WOUND HEALING IN NORMAL AND SCORBUTIC GUINEA-PIGS

by

CLAËS VON NUMERS

(Received for publication September 16, 1953)

The ground substance of the mesenchymal tissues contains various mucopolysaccharides combined with proteins. In the connective tissue of the corium the presence of at least two mucopolysaccharides has been established, *viz.* hyaluronic acid and chondroitin-sulphuric acid. Whilst metachromatic substance is present in abundance in foetal skin, little or none is demonstrable by ordinary histochemical methods in the normal corium of full-grown organisms. The external sheaths and the papillae of growing hair follicles seem to be the tissues richest in mucopolysaccharides (6, 10). For further details the reader is referred to Asboe-Hansen's (1) paper of 1951, in which the relevant investigations are reviewed at length. Asboe-Hansen has studied the occurrence of metachromatic substance in the skin both under normal conditions and in various skin lesions, and in certain cases of hormonal disturbance. With regard to general changes in the ground substance of the connective tissue in scurvy, which are not dealt with at all in Asboe-Hansen's (1) paper, a survey of the meagre data available is given by Gersh (5) in the *Transactions of the Second Connective Tissue Conference*, May 1951. Scurvy has proved to be one of

the diseases in which the ground substance of the connective tissue becomes markedly depolymerized and, finally, water-soluble. Hence the molecules of the decomposition products become small enough to enter the blood, with a resulting increased plasma mucoprotein level. As the chemical composition of collagen is largely normal in scurvy, ground substance is formed continually and passes more or less directly into the circulation. It has been stated that the connective tissue in scurvy is metachromatic and has returned to a more foetal state.

The role of vitamin C in the regeneration of connective tissue in wound healing is much better known. Wohlbach (14) showed that a proliferation of fibroblasts took place in scorbutic guinea-pigs in connection with wound repair; the new cells did not, however, possess the capacity to form fibrils. This function of the connective tissue cells was promptly restored after administration of orange juice. Wohlbach's observations were later confirmed by other investigators. It was established by Crandon (3) that vitamin C is necessary for the formation of intercellular substance in the human organism also. Ingalls (8) and Bourne (2) have shown that the tensile strength of healing wounds is decreased in guinea-pigs suffering from partial vitamin C deficiency. Nylander (11) found that the formation of fibrils was imperfect in the omentum of scorbutic guinea-pigs in the foreign body reaction. In scorbutic animals the architecture of healing wounds is disturbed; the cells remain immature and their formation of collagen is inadequate. The granulation tissue is poorly developed and haematomata which do not become organized occur owing to the fragility of the capillaries. The healing of abscesses is delayed in animals suffering from vitamin C deficiency (9). The necrotic centre is not walled off and the number of macrophages is decreased. In 1949, Penney and Balfour (12) examined the production of mucopolysaccharides in wound repair. In scorbutic guinea-pigs little or no metachromatic substance was found in the granulation tissue, the cells of which were morphologically abnormal. In the controls, mucopolysaccharides were demonstrable in considerable amounts during the reparative process, and the same was found in scorbutic animals as early as 12 hours after injection of ascorbic acid.

In connection with recent investigations on cells and substances displaying metachromatic staining the writer became interested

in the question of mucopolysaccharides in the skin in scurvy. The mast cell was chosen as the object of study since, to the best of the writer's knowledge, it has not previously been examined from this point of view.

Material and Methods. — These investigations were carried out at the Department of Medical Chemistry of Helsinki University, the Head of which, Professor P. Simola, M.D., Ph.D., kindly placed working facilities at the writer's disposal. The series consisted of 18 guinea-pigs, initially weighing from 450 to 600 g. They were weighed once a week during the course of the investigation. The animals were used as follows:

Group 1. Seven guinea-pigs were given the vitamin C free diet composed by Simola (13). The loss in weight during the experiment varied from about 8 to 30 per cent, the average being about 23 per cent. On the 13th day five animals were operated on in the same manner as in Wohlbach's experiments, a large piece of the cutis, subcutis and extensor thigh muscle being removed under anaesthesia. These animals were sacrificed and the wound area was excised on the 7th to 9th day after operation. In one case the operation was performed on the 16th day. This animal died spontaneously four days after operation, and the wound area was excised about half an hour *post mortem*. One animal was not operated on; it died spontaneously on the 19th day, and a sample of the same kind as those obtained by operation in the other cases was taken less than one hour *post mortem*.

Group 2. This group, too, included seven guinea-pigs which were given the same vitamin C free diet. Four animals were operated upon on the 14th day and given 10 mg of ascorbic acid from the fifth day after the operation. They were sacrificed one, two, or three days after the beginning of the ascorbic acid medication. The three remaining animals were operated upon on the 16th day; administration of vitamin C was begun seven days later, and the animals were sacrificed one, two and five days after treatment had commenced.

Group 3 consisted of four controls, which were given a standard diet containing sufficient amounts of vitamin C. The above-mentioned operation was performed on these animals also. They were sacrificed and the wound area was excised on the 6th, 7th, 8th and 9th day, respectively, after the operation.

The following techniques were employed in the fixation and staining of the samples of tissue and the wound areas obtained:

fixation in 10 per cent formol, staining by van Gieson's method and with haematoxylin and eosin;

fixation in a 4 per cent aqueous basic lead acetate solution and staining with toluidine blue by the method employed by Holmgren and Wilander;

fixation in Carnoy's solution, staining by the Azan modification of Mallory's method for reticulin fibrils and by the method used by McManus and Hotchkiss (PAS) for mucopolysaccharides.

One of the main purposes of the present investigation was to ascertain whether mast cells occur in scorbutic animals and, if present, differ from the normal in number. Floderus's formula was therefore used for the determination of the content of these cells per unit volume of tissue. For details regarding this formula the reader is referred to Floderus's paper of 1944 (4) or to Hjelmman's (6) investigations on mast cells in the human embryo, in which this formula was used.

The mast cells in the cutis occur chiefly in the subepithelial layer. On counting them (or rather the number of mast cell nuclei), 10 sq mm of each preparation were studied. This area included the tissue extending from the border of the epidermis to a depth of four fields. The preparations were 5 μ thick. The average size of the mast cell nucleus was estimated at 4 μ, and the shrinkage of the tissue resulting from fixation at 10 per cent by volume. Zeiss's objective No. 40 and No. 15 oculars were used.

RESULTS

Free Mucopolysaccharides of the Intact Skin. — In samples of subepidermal connective tissue from normal animals, stained with toluidine blue, little or no free metachromatic substance was discovered. In two cases the results were negative, in the other two a very light, reddish tint was observable. Examination of preparations from the 14 scorbutic guinea-pigs gave largely the same results, only more markedly negative, perhaps, very light metachromatic staining being observable in two cases only. In PAS preparations the corium always stained light red, whilst a narrow, bright red zone was observable in the connective tissue adjacent to the epidermis. In this respect there was no difference between the preparations from normal and scorbutic animals.

In toluidine blue preparations the epidermis from the four normally fed guinea-pigs stained altogether orthochromatically; it was fairly thin, and the nuclei were conspicuous (Fig. 1). The epidermis from the animals suffering from vitamin C deficiency displayed, as a rule, marked deviations from this normal structure. It appeared to be somewhat swollen and thickened, although no proliferation of cells, with resultant increase in the number of cell rows, was discernible. The outlines of the nuclei were somewhat blurred, and the whole of the epidermis displayed a marked metachromatic staining (Fig. 2). In 10 of the 14 experimental animals these changes were marked, whilst slight changes were observed in 2. Only in 2 animals were no changes demonstrable. In PAS

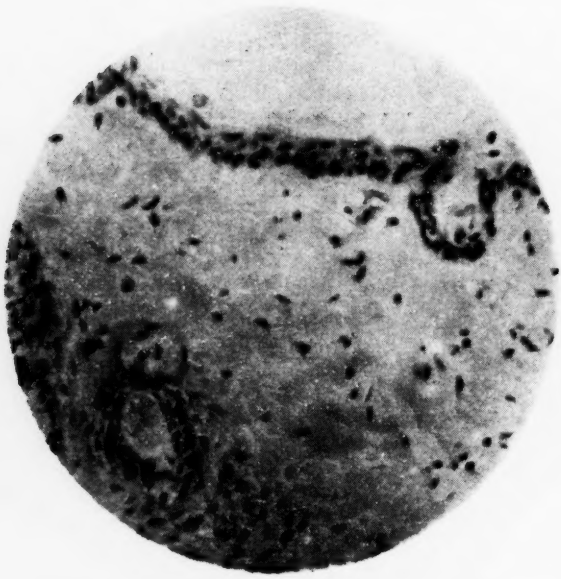

Fig. 1. — Normal guinea-pig. Epidermis pale, orthochromatically staining. Basic lead acetate, toluidine blue. × 190.

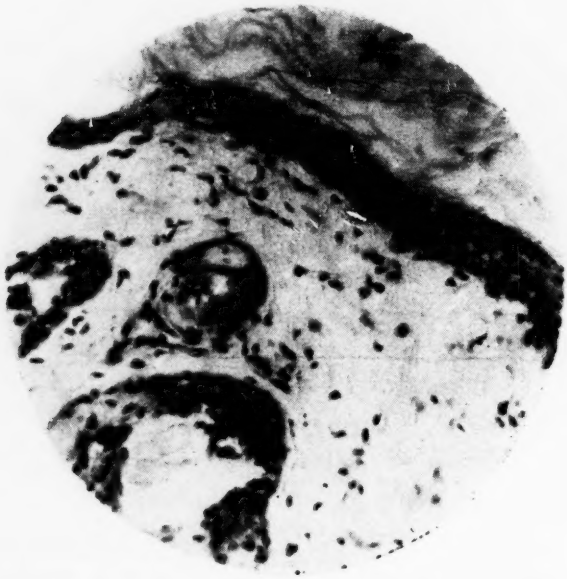

Fig. 2. — Scorbutic guinea-pig. Owing to metachromatic staining the epidermis appears dark. Basic lead acetate, toluidine blue. × 190.

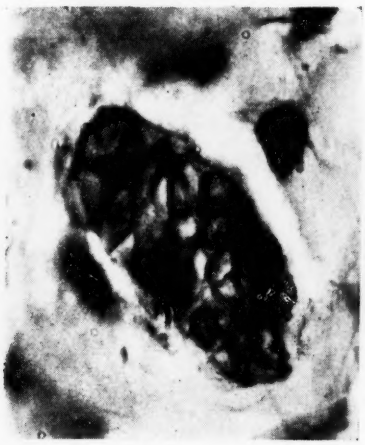

Fig. 3. — Normal guinea-pig. Cross-section of arrector pili muscle. Carnoy, PAS + hematoxylin. × 1300.

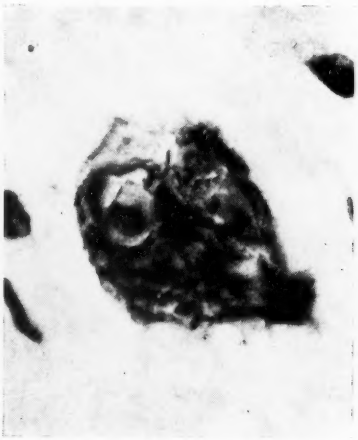

Fig. 4. — Scorbutic guinea-pig. Cross-section of arrector pili muscle. Carnoy, PAS + hematoxylin. × 1300.

preparations no positive staining of the epidermis was obtained either in the controls or in the experimental animals.

Of the epidermal appendages, the hair follicles were especially studied. In toluidine blue preparations from normal animals metachromatic staining was observed in the connective tissue of the papilla, in the cells of the external sheath, and in the connective tissue surrounding the hair follicle. This is in agreement with previous observations. On the other hand most hair follicles stained orthochromatically throughout, whilst only a few bulbs, obviously belonging to growing hairs, displayed the above-mentioned features. In some hair follicles the above-mentioned parts stained positively by the PAS method, as was to be expected. Furthermore numerous, fairly large, markedly red-staining granules were discernible distally from the hair bulb in the cells of the external sheath. These granules probably consisted of glycogen.

Examination of hairs and hair follicles from scorbutic animals did not reveal any abnormal features. These were observable, however, in PAS preparations of muscle fibres from the arrectores pilorum. In normal cases the fibres displayed very small, red-staining granules, indicative of the presence of muscle glycogen, and localized solely in the periphery (Fig. 3). In the scorbutic guinea-pigs the red colour was unevenly spread over the whole of

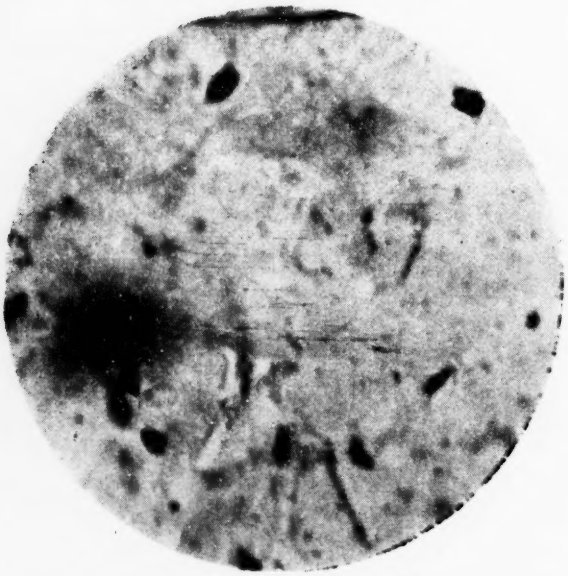

Fig. 5. — Mast cell with few granules, surrounded by a diffuse area of free mucopolysaccharides. Basic lead acetate, toluidine blue. × 750.

the fibril, and coarser granules of glycogen were observed here and there (Fig. 4). Hence the regular, mosaic-like structure characteristic of the normal arrector pilii muscle was more or less disturbed.

Mast Cells of the Intact Skin. — As has been mentioned, the mast cells of the corium of the guinea-pig are, as a rule, found in the juxta-epithelial layer. In the deeper parts they are much sparser and mostly situated perivascularly. From the morphological viewpoint the mast cell of the guinea-pig does not differ essentially from the human mast cell. The granules of the former seem, however, to be smaller and more dispersed in the plasma. A release of metachromatic granules is sometimes observed in the guinea-pig also. Not infrequently a diffuse, metachromatically staining zone surrounds the mast cell of the guinea-pig like a halo (Fig. 5). It seems as if metachromatic substance had been released from disintegrating granules and spread to the surroundings of the cell. No differences between normal and scorbutic guinea-pigs were discovered with regard to the morphological properties of the mast cell and the phenomena of extrusion and diffusion of metachromatically staining matter.

A mast cell count, carried out by the method described above, gave the following results:

In the controls the number of mast cells per cu mm of connective tissue varied from 1,350 to 1,910, the average being 1,630 cells.

In the animals fed on a vitamin C free diet the corresponding figures were 225, 1,350 and 626.

In this connection it should be mentioned that mast cells were also demonstrable in the guinea-pigs that died from absolute scurvy during the experiment, although in small numbers (225 cells/cu mm).

Free Mucopolysaccharides of the Healing Wound and its Surroundings. — Earlier observations on the granulation tissue of healing wounds in scorbutic guinea-pigs were confirmed by the present investigation. The proliferative potency of the fibroblasts appeared to be somewhat impaired. It was established from Azan preparations that newly formed fibrils were absent, and from toluidine blue preparations that the growing tissue did not display any metachromasia. On PAS staining, red granules, varying in size, were observed in large numbers in the wound cavity together with the disintegrating cells. In the connective tissue adjacent to the wound area mucopolysaccharides were not demonstrable.

The process of wound repair seemed to be normal both in the controls and in the animals treated with vitamin C, formation of fibrils and metachromatically staining substance in the granulation tissue being demonstrable. Metachromasia was also observed in the surrounding parts of the corium.

Mast Cells of the Healing Wound and its Surroundings. — The granulation tissue proper was found to be poor in mast cells. In the scorbutic animals their number varied between 110 and 450 per cu mm, the average being 224 cells. The corresponding figures for the non-scorbutic animals were 340, 1,010 and 590.

In three of the controls, mast cells with a nucleus relatively poor in chromatin and with very small, faintly stainable granules were encountered in the granulation tissue. It seems probable that these mast cells were immature and newly formed. Such forms were not observed in the other guinea-pigs.

The portions of the corium immediately surrounding the wound area were examined for the content of mast cells on the same

principles as were followed in the study of the intact skin. The following results were obtained:

Scorbutic animals 225—2,250 cells, average 1,215 cells,
Treated animals 785—2,025 cells, average 1,312 cells,
Control animals 2,590—5,060 cells, average 3,769 cells.

DISCUSSION

At least within the area from which samples were taken for the present investigation, the corium of the normal guinea-pig is poor in mucopolysaccharides demonstrable by the usual histochemical methods. A more marked metachromasia in scorbutic guinea-pigs, which might have been expected in view of certain previous observations (5), was not established in any instance. On the contrary, weak positive reactions were even rarer than in the controls. This was perhaps contingent on the fact that the present investigation was performed on animals suffering from absolute scurvy, which obviously results in a maximal depolymerization of mucopolysaccharides. The same is indicated by the changes in the epidermis observed in the scorbutic animals. Both the swelling of the epidermis and the metachromatic staining are probably attributable to imbibition of depolymerized, evidently water-soluble mucopolysaccharides, which subsequently become repolymerized. Whether this transport of mucopolysaccharides occurs directly or via the circulating blood is difficult to ascertain. The former alternative seems more likely.

With regard to the epidermal appendages it is a noteworthy fact that vitamin C deficiency did not cause any demonstrable changes in the hair follicles themselves nor any decrease in the relative number of growing, metachromatically staining follicles. The change in the structure of the arrectores pilorum observable in PAS preparations is undoubtedly ascribable to disturbances in the carbohydrate metabolism of the muscle fibrils. Thus experimental scurvy has no evident inhibitory influence on hair growth. Hence there is no noteworthy loss of hair in scorbutic guinea-pigs.

The mast cell of the guinea-pig is frequently surrounded by a diffuse, metachromatically staining area. The occurrence of mast cells in the corium of the intact skin is obviously influenced by

vitamin C deficiency. Although there was a marked decrease in the number of mast cells, these did not disappear completely from the connective tissue even when the experimental animal died from absolute scurvy. Obviously the position is the same as that reported by Meyer and Meyer (cf. above) with regard to macrophages.

The absence of newly formed fibrils and free, metachromatically staining mucopolysaccharides in the granulation tissue in scurvy has been established by previous investigators. In this connection the present writer only wishes to emphasize the abundant occurrence of PAS positive granules in the wound cavity. These seem to consist, in part at least, of glycogen or decomposition products of glycogen. The latter also stain positively with periodic acid.

The granulation tissue of the healing wound was found to be relatively poor in mast cells both in normal animals and in those suffering from vitamin C deficiency. There was no appreciable difference, even though the number of mast cells was as a rule somewhat lower in the scorbutic animals. On the other hand a marked increase in the number of these cells was observed in the areas of the corium adjacent to the healing wound. This reaction was quantitatively much stronger in the connective tissue of normal guinea-pigs. In these, immature forms of the mast cell occurred, moreover, indicating the formation of new cells.

The capacity of the connective tissue to form fibrils and highly polymerized mucopolysaccharides is absolutely dependent on a sufficient supply of ascorbic acid. On the other hand the occurrence of mast cells in the connective tissue of the corium and their accumulation in connection with wound repair is only to some extent dependent on the presence of vitamin C, just as are the occurrence of macrophages and the capacity for proliferation of the fibroblasts.

SUMMARY

Metachromatically-staining mucopolysaccharides are of rare occurrence in the corium of the normal guinea-pig and do not display any increase in absolute scurvy.

Vitamin C deficiency causes a deposition of free mucopolysaccharides in the epidermis.

The relative frequency of growing hair follicles and their content of metachromatically staining substances do not undergo any demonstrable change in scorbutic animals. In PAS preparations disturbances in the fibrils of the arrectores pilorum were revealed.

In the corium of the intact skin of animals suffering from scurvy the number of mast cells was decreased.

The process of wound repair entails an increase in the number of these cells in the surrounding parts of the corium. This reaction is, however, quantitatively much weaker than in normal guinea-pigs.

REFERENCES

1. Asboe-Hansen, G.: Om bindevævets mucinøse substanser (Mucinous Substances of Connective Tissue). — Thesis. Copenhagen 1951.
2. Bourne: cit. by Bicknell, F., and F. Prescott: The Vitamins in Medicine. — London 1947. W. Heinemann.
3. Crandon: ibid.
4. Floderus, S.: Acta path. et microbiol. Scand. 1944:*53*, Suppl.
5. Gersh, I.: Connective Tissues. — Transact. Second Conf. May 24.—25., 1951.
6. Hjelmman, G.: Comment. Biologiae Soc. Scient. Fenn. 1952:*13*:1.
7. Holmgren, H.: Zschr. f. mikr.-anat. Forsch. 1940:*47*:489.
8. Ingalls: cit. by Bicknell, F., and F. Prescott: The Vitamins in Medicine. — London 1947. W. Heinemann.
9. Meyer, E., and M. B. Meyer: Bull. Johns Hopkins Hosp. 1944:*74*:98.
10. Montagna, W., H. B. Chase and Helen P. Melaregno: Journ. Nat. Canc. Inst. 1951:*12*:591.
11. Nylander, P. E. A.: Acta Soc. Med. Fenn. Duodecim, Ser. A, 1939: *22*:30.
12. Penney, J. R., and Brigid M. Balfour: J. Path. Bact. 1949:*61*:171.
13. Simola, P.: Acta Soc. Med. Fenn. Duodecim, Ser. A, 1932:*16*.
14. Wolbach, S. B.: Am. Journ. Path. 1933:*9*:689.

FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

HISTOLOGICAL CHANGES IN THE LIVER TISSUE OF THE WHITE RAT

DURING REGENERATION AND AFTER INTRA-ABDOMINAL TRANSPLANTATION

by

CLAËS VON NUMERS and P. FORTELIUS

(Received for publication September 24, 1953)

The great capacity of the liver for regeneration has been known since the end of last century (2, 4, 8). The time required for this process varies somewhat with the species and age of the experimental animals. It should, however, be borne in mind that not all the results are comparable because different criteria have been used in the evaluation of the degree of regeneration (8, 11). After removal of about 70 per cent of the liver of the white rat, the weight of the remaining part is doubled within 24 hours. About 80 per cent of the initial weight is regained within a week, and, moreover the total number of cells almost attains the initial figure in the same time (1). Liver function, too, is nearly restored within a week after excision (11). The rate of mitosis is highest on the second to third day (1, 3). Thus it may be stated that the growth of the tissue here concerned is very rapid indeed.

According to previous investigations, of which those of Sylvén (10) may be mentioned, rapid growth is accompanied, as a rule, by a marked metachromatic reaction of the connective tissue. Metachromasia is due to an increased content of high molecular mucopolysaccharides. As is well-known, the granules of the mast cells are also made up of such substances.

In connection with a series of investigations on metachromatic substances (5, 6), regenerating liver tissue was examined for the occurrence of free mucopolysaccharides and mast cells. This tissue was considered a suitable object of study in view of its rapid growth.

Furthermore, pieces of normal and of regenerating rat liver were implanted into the peritoneal cavity of other rats for the purpose of ascertaining whether regenerating liver possesses a relatively greater vitality. In connection with these experiments the reaction of the omentum and the invasion of cells into the implant were studied from the viewpoint of a possible occurrence of metachromasia and mast cells.

Methods and Results. — Fullgrown white rats were used as experimental animals. A part of the liver was removed by a method described by Brues and Drury & Brues (1). Specimens were taken from the regenerating liver tissue $4\,^1/_2$ hours to 8 days after operation. The pieces of liver used in the implantation experiments were the size of a split pea and smaller. These were always taken from the liver of newly-killed rats, and placed immediately in sterile saline solution at about body temperature. They were transplanted as soon as possible into the peritoneal cavity of normal rats. The operation, which was performed under ether narcosis, consisted of a small mid-line incision, through which the piece of liver was inserted. The wound was closed in two layers with a continuous silk suture, and painted with collodium. The operated animals were killed and the implants removed for microscopical examination from $6\,^1/_2$ hours to 4 days after transplantation. Preparations from each regenerating, or implanted, liver were fixed in formalin and stained by v. Gieson's method, fixed in lead acetate and stained with toluidine blue in an aqueous solution, or fixed in Carnoy's solution and stained by the Azan modification of Mallory's method. Toluidine blue staining was performed with a 5 per cent aqueous solution and followed by dehydration in absolute alcohol to eliminate the occurrence of false metachromatic reactions.

The histological picture of normal liver varies according to the content of glycogen, fat, biliary pigment, etc. The variations seen in preparations of regenerating liver are still greater, owing partly to differences in the degree of regeneration. It was observed in our preparations from regenerating liver tissue that, during the first 24 hours, the nucleus was large and pale, and often had a fairly

large nucleolus. Later the nuclei seemed to be smaller and darker. Considerable variations occurred, however, and the same was true of the cytoplasm, which was abundant and pale during the first day. Most preparations revealed hyperaemia, which was often marked, and inflammatory infiltration varying in degree. *No metachromasia could be established either in the periportal areas or in the reticulum of the regenerating parenchyma. Neither was any increase in the number of mast cells observable.*

The pieces of liver implanted into the peritoneal cavity were always adherent to the omentum and to the intestinal serosa. The longer the period that had elapsed after implantation, the firmer were the adhesions. Only 6 hours after implantation, three different concentric zones were clearly distinguishable here and there in the implants (Fig. 1). In the outermost zone there were only faintly stained remnants of liver cells without nuclei. The next layer consisted of tissue that was highly infiltrated by blood cells and macrophages migrating towards the interior along tissue spaces, capillaries and greater vessels (Fig. 2). The innermost zone was made up of liver parenchyma in varying stages of autolysis. The nuclei were often pyknotic. In preparations from 24-hour-old implants the peripheral layer was, as a rule, clearly distinguishable; cells with intact nuclei were rare (Fig. 3). The phagocyte wall consisted mostly of granulocytes and macrophages, the latter of which contained dark granules in abundance. In the central zone most nuclei were pyknotic, but karyorrhexis and karyolysis also occurred (Fig. 4). The older the transplant, the more diffuse was the cell structure. *No difference between transplants of normal and of regenerating liver could be established.* The reaction of the omentum was marked, and in preparations from the older transplants an increase of the fibrils, too, was demonstrable by Azan staining. Furthermore metachromasia, increasing with the age of the transplant, was observed in toluidine blue preparations from the surrounding parts of the omentum. It is a very noteworthy fact that mast cells occurred in large numbers even in the earliest preparations, in the periphery of the transplants (Fig. 5). They were mostly localized to the border zone between the implant and the adhering omentum. Mast cells were seen in the leukocyte wall, too, and occasionally inside it, though never in large numbers.

Discussion. — Yokoyama *et al.* (11), using mouse liver, dem-

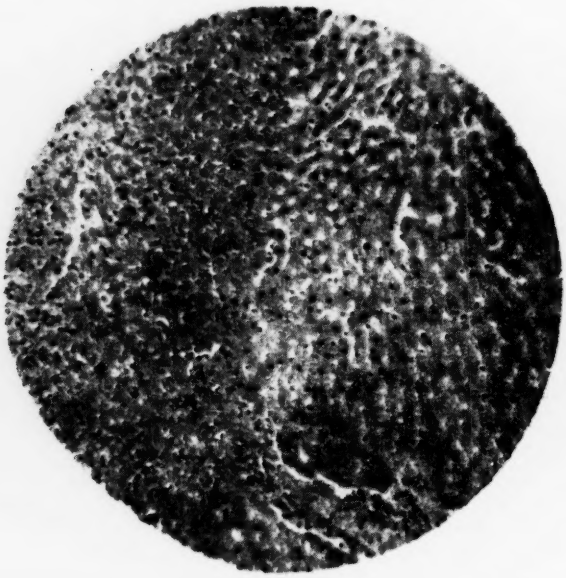

Fig. 1. — Necrotizing liver tissue. Outermost zone to the left. Haematoxylin + eosin. × 190.

Fig. 2. — Inflammatory infiltration along capillaries and a greater blood vessel. Haematoxylin + eosin. × 190.

Fig. 3. — The three zones of necrotizing liver tissue. To the left proliferating omentum adherent to the transplant. Haematoxylin + eosin. × 30.

Fig. 4. — Liver tissue from the central zone. Pyknosis predominant, karyolysis and karyorrhexis to some extent. Haematoxylin + eosin. × 560.

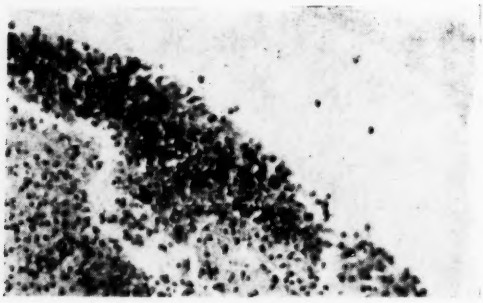

Fig. 5. — Periphery of necrotizing transplant. Mast cells in abundance. Toluidine blue. × 90.

onstrated by chemical and histochemical methods that a great deal of the gain in weight during the first 24 hours is due to a mobilization of lipoids to the parenchymal cells and to an increased liquid content. According to Higgins & Anderson (3), in rats, too, the growth of the liver during the first day is ascribable to hypertrophy of the nuclei and the cytoplasm. This conclusion is in accord with our observations. These authors found that mitosis set in towards the end of the first day; during the second and third day the rate was high, after which the growth rate and rate of mitosis were both low until the seventh day, when an increase in mitotic activity occurred simultaneously with a gain in weight. Hence it would seem as if the growth of regenerating liver were cyclic. Brues, Drury & Brues (1) found that the increase in the number of nuclei was slower than the increase in weight; they established by micrometry that both the cells and the nuclei were larger than normal during regeneration. The light colour macroscopically discernible in regenerating liver appears under the microscope as a paler tint in the parenchymal cells. This is attributed by Ponfick (8) to a larger content of protoplasm and a smaller content of biliary pigment than in normal liver. According to Yokoyama (11) the nucleolus is reduced in size during the first day and grows at the time of increased mitotic activity, when the protein synthesis is probably increased.

No definite increase in the number of mast cells nor any marked metachromasia were observed in our preparations. This is to some extent in conflict with certain earlier observations which pointed to an abundant occurrence of mucopolysaccharides and mast cells in rapidly growing tissues.

Reporting on experiments involving transplantation of pieces of liver from rats into the peritoneal cavity of other rats, Rössle (9) described the devision into three layers referred to above, which is observable in preparations from such implants. According to him, the three separate layers are only distinguishable after 72 hours. In our preparations this division appeared after only 6 hours, although it was not visible everywhere. Rössle observed surviving liver cells in the outermost layer as late as 48 hours after implantation, and described rows of such cells with fairly intact nuclei in the peripheral zone during the first day. As was pointed out above, we were not able to detect any surviving liver cells in the outermost layer even after 6 hours. The disintegration of the outermost layer was attributed by Rössle to heterolysis, *viz.* to the effect of substances produced by the host animal. It is obvious that this necrosis of the peripheral layer is much more rapid than the corresponding process inside the leukocyte wall (*viz.* autolysis). The accumulation of mast cells around the implant observed in our preparations was doubtless due to invasion from the omentum. In the necrotic implants mast cells were only occasionally encountered. Nylander (7), who studied the inflammatory reactions of the omentum, did not observe mast cells in any appreciable number. He was, however, concerned with bacterial infections whereas the reaction of the omentum in our experiments was brought about by implants that were as sterile as possible. The metachromasia of the omentum is due to a proliferation of cells involving an accumulation of mucopolysaccharides. It seems probable that substances are released from the necrotic implant which have a positive chemotactic effect on the mast cells. The etiology is, perhaps, essentially the same as in thrombosis, where mast cells accumulate around the thrombosed vessel.

SUMMARY

Regenerating liver tissue was studied with special reference to the occurrence of metachromasia and mast cells. No marked increase could be established in either. Furthermore experiments were performed involving transplantation of normal and of regenerating liver. No difference in the histological pictures was observed. A marked metachromasia and a large accumulation of mast cells in the surrounding omentum were noted.

REFERENCES.

1. BRUER, A. M., D. R. DRURY and M. C. BRUER: Arch. Pathol. 1936: *22:*658.
2. CRUVEILHEIR: cit. by HIGGINS and ANDERSSON.
3. HIGGINS, G. M., and R. M. ANDERSSON: Arch. Pathol. 1931:*12:*186.
4. v. MEISTER: cit. by BRUER, DRURY and BRUER.
5. v. NUMERS, C.: Annal. med. exp. et biol. Fenn. 1953:*31.* In press.
6. v. NUMERS, C., and P. FORTELIUS: Ibid. In press.
7. NYLANDER, P. E. A.: Arbeit. aus d. Path. Inst. Univ. Hels. N. F. 1933:*7:*453.
8. PONFICK, E.: Virch. Arch. 1894:*138:*81.
9. RÖSSLE, R.: Sitzungsber. d. Preuss. Akad. d. Wissensch., phys.-mathem. Klasse 1936:22.
10. SYLVÉN, B.: Acta Radiolog. 1945, Suppl. 59.
11. YOKOYAMA, H. O., M. E. WILSON, K. K. TSUBEI and R. E. STOWELL: Canser Res. 1953:*13:*80.

FROM THE DEPARTMENT OF PATHOLOGY, UNIVERSITY OF HELSINKI

CUTANEOUS AND SUBCUTANEOUS CHANGES IN MICE

CAUSED BY PERCUTANEOUS ADMINISTRATION OF THE ASSOCIATION COLLOID TWEEN 80, WITH AND WITHOUT HEPARIN

PRELIMINARY REPORT

by

CLAËS von NUMERS and KAI SETÄLÄ

(Received for publication September 30, 1953)

The quantitative variations of the free mucopolysaccharides and the behaviour of the mast cells in different tumours and in the tissues during certain pathological conditions have previously been studied by one of the present writers (4, 5, 6, 7). Using association colloids as solvents for carcinogenic hydrocarbons in a series of investigations, Ekwall and Setälä *et al.* (1, 2) demonstrated the ability of these solutions to penetrate the skin and other tissues. Since these solvents are capable of dissolving both water- and lipid-soluble substances, experiments were undertaken to ascertain whether heparin, dissolved in a suitable association colloid and percutaneously administrated, brings about histologically demonstrable changes in the content of free mucopolysaccharides and mast cells in the subcutaneous tissue. For this purpose a solution was prepared of 0.5 per cent heparin (moisture content 14.8 per cent, activity 91 units per mg.dry) in Tween 80 (polyoxyethylene sorbitan monooleate, hydrophile-lipophile balance 15.0). During the course of the work it was found that Tween 80 by itself caused changes deserving of particular attention.

The above-mentioned solution was painted on the back of adult white stock mice. Biopsies from the treated area of the skin were

fixed in a 4 per cent basic lead acetate solution and stained with a 0.5 per cent toluidine blue aqueous solution and by van Gieson's method. In counting the number of the mast cells, Floderus's formula (3) was used.

The mice used in the experiments were divided into the following series, each of which included 10 animals:

Series 1 (mice Nos. 1—10). Untreated controls.
Series 2 (mice Nos. 11—20). Heparin — Tween 80 solution one painting. Biopsy two hours later.
Series 3 (mice Nos. 21—30). Heparin — Tween 80 solution two paintings a day, total number of paintings four. Biopsy three hours after last painting.
Series 4 (mice Nos. 31—40). Heparin — Tween 80 solution two paintings a day, total number of paintings five. Biopsy three hours after last painting.
Series 5 (mice Nos. 41—50). Heparin — Tween 80 solution two paintings a day, total number of paintings eight. Biopsy six hours after last painting.
Series 6 (mice Nos. 51—60). Tween 80 one painting. Biopsy two hours later.
Series 7 (mice Nos. 61—70). Tween 80 two paintings a day, total number of paintings five. Biopsy three hours after last painting.
Series 8 (mice Nos. 71—80). Tween 80 two paintings a day, total number of paintings eight. Biopsy six hours after last painting.

The calculations of the number of mast cells per cubic millimetre of tissue gave the following results (arithmetical means for each series):

Series 1	14,052	cells/cu mm	
» 2	15,695	»	»
» 3	7,898	»	»
» 4	5,101	»	»
» 5	6,558	»	»
» 6	10,283	»	»
» 7	5,888	»	»
» 8	6,773	»	»

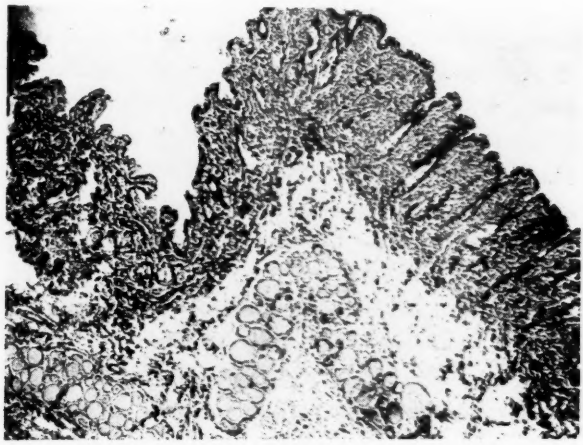

Fig. 1. — Skin of untreated animal. Mouse No. 10, Series 1. Basic lead acetate, van Gieson. × 50.

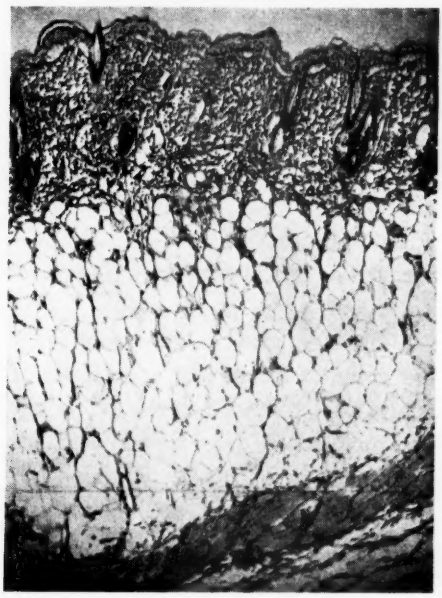

Fig. 2. — Skin after four paintings with heparin-Tween 80 solution. Mouse No. 24, Series 3. Basic lead acetate, van Gieson. × 50.

Slight histological changes or none were observable in the tissues examined from Series 2. All preparations from Series 3, on the other hand, displayed marked deviations from the normal The subcutaneous adipose tissue, which was very scanty in the untreated controls (Fig. 1) had increased many times in bulk as a result of the treatment (Fig. 2). This was chiefly due to enlargement of the fat cells themselves. Furthermore the cutaneous connective tissue had undergone a change; it was less dense than before, the spaces between the fibrils having increased both in size and number. No free metachromatic substance was demonstrable anywhere. A striking feature was the occurrence of a large number of small mast cells with few granules. Moreover, many mast cells contained granules in which the staining was lighter than normal. Extruded granules occurred in abundance.

The preparations from Series 4 showed the same changes as those from Series 3.

The biopsies from Series 5 displayed certain changes which differed markedly from those described above. The fibrils of the cutaneous connective tissue were much thicker than normal, swollen, and markedly metachromatic. The same features were observed in the connective tissue bands in the adipose tissue; the latter was as thick as in the preparations from Series 3 and 4. The mast cells appeared, on the whole, to be normal, small cells with scanty granulation were present in decreased number.

In Series 6, 7 and 8, in which Tween 80 alone was used, the same changes were observed as in the corresponding series where heparin heparin had been added.

Discussion. — The decrease in the number of mast cells was doubtless to some extent relative and contingent on the expansion of the cutaneous and subcutaneous tissues resulting from the treatment. Nevertheless an absolute decrease seems also to have occurred. The morphological features of the mast cells in the preparations from Series 5, and the fact that they had increased somewhat in number, seem to indicate that the position had again become stable with regard to these cells.

The very noteworthy changes described above in the subcutaneous adipose tissue, in particular, are doubtless directly due to the penetration of the association colloid into the fat cells. The resulting intracellular coalescence with the cytoplasmic fat sub-

stances obviously results in enlargement and expansion of the fat cells. Since similar effects were obtained also without the use of heparin, the reactions of the mast cells, too, must be chiefly due to the association colloid. The strong metachromatic reaction in Series 5 was perhaps brought about by mucopolysaccharides released from mast cell granules, and polymerized and precipitated in the cutaneous and subcutaneous connective tissue during the later stages of the treatment.

The present findings seem to shed new light on the percutaneous penetration of certain compounds and to provide a new approach to the study of mucopolysaccharide metabolism.

SUMMARY

Percutaneous administration of the association colloid Tween 80 caused a noteworthy increase in the bulk of the subcutaneous adipose tissue and a considerable enlargement of its cells. A decrease in the number of mast cells and morphological changes in these cells were observed. After somewhat more prolonged treatment (2 paintings a day for 4 days) the connective tissue fibrils were much thicker than normal and showed a marked metachromatic reaction.

Addition of heparin to the association colloid did not influence the results.

REFERENCES

1. Ekwall, P., and K. Setälä: Acta Chem. Scandinav. 1948:*2:*733.
2. Ekwall, P., P. Ermala and K. Setälä: Cancer Res. 1951:*11:*758.
3. Floderus, S.: Acta Path. et Microbiol. Scandinav. 1944, Suppl. 53.
4. v. Numers, C.: Acta Obst. et Gyn. Scand. 1942;*2*, Suppl.
5. v. Numers, C.: Annal. Med exp. et Biol. Fenn. 1953:*31:*199.
6. v. Numers, C.: Ibid. 1953:*31.* In press.
7. v. Numers, C., and P. Fortelius: Ibid. 1953:*31.* In press.

FROM THE RHEUMATISM FOUNDATION HOSPITAL, HEINOLA, AND FROM THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF HELSINKI

THE EFFECT OF CHLOR-TRIMETON AND THEPHORIN ON THE CIRCULATORY AND BLOOD SUGAR CHANGES PRODUCED BY ADRENALINE [1]

by

M. J. OKA and M. K. PAASONEN

(Received for publication October 10, 1953)

In animal tests antihistaminics, phenergan in particular, block adrenaline pulmonary edema (4, 11—13, 21, 24, 29). Negative results are not lacking (25, 30). In our experiments (20) we were able to show that there were two groups of antihistaminics, of which some such as chlortrimeton, benadryl, anthisan, and pyribenzamine increased the adrenaline toxicity. On the other hand thephorin and phenergan and, to some extent antistine, increased the LD of adrenaline. It was shown by Tislow *et al.* (28) that chlortrimeton increases the pressor effect of adrenaline. Lehmann *et al.* (17) showed in blood pressure tests the anti-adrenaline effect of thephorin. In the literature we notice that also other antihistamines used in our earlier experiments, which increase adrenaline toxicity, are inclined to increase adrenaline blood pressure response and *vice versa*.

The blood sugar increasing ability of adrenaline is slightly potentiated by benadryl (1). Chlor-trimeton increases and thephorin weakenes the adrenaline response in dogs (26). Komrad and Loew (16) were able to show that antihistaminics block adrenaline hyperglycemia in rabbits. However, chlor-trimeton among others poten-

[1] We wish to thank Schering Corp., Bloomfield, N. J., for »Chlor-trimeton» (chlorprophenpyridamine maleate) and F. Hoffmann-La Roche & Co., Basle, for »Thephorin» (phenindamine hydrogen tartrate).

tiated the adrenaline effect in dogs. In our tests made with rats (20) neither chlortrimeton nor thephorin could be shown to have any clear effect on the hyperglycemic ability of adrenaline. According to Scheidhauer (23) antistine *per os* prevents adrenaline hyperglycemia in children.

Since allergic conditions are the main indications for the use of antihistaminics, it is obvious that adrenaline, which is the most potent known anti-allergicum, often comes to be given with them at the same time (9). On the basis of animal experiments it was expected that antihistamines have some effect upon adrenaline responses in human beings also. As typical representatives of the two antihistamine groups possessing different sympathetic characteristics, chlor-trimeton and thephorin were chosen, and changes of blood pressure, heart rate and blood sugar were measured.

PRESENT INVESTIGATION

The series consisted of 15 cases from the Hospital for Rheumatic Diseases 12 of them were male patients and the remaining three from the woman staff. Only those patients whose general condition was good and whose joint symptoms were as slight as possible were chosen. Ten of these were suffering from rheumatoid arthritis and two from spondylarthritis anchylopoetica. In one of the female cases an allergy appeared in the case-history. The age of the cases was 22—52 years (average 34.1), the weight 47.5—86.3 kg (average 67.8). The sedimentation rate of the rheumatic patients was 2—63 mm hr. (average 20 mm hr.).

All the tests were made in the mornings. The test patients were without food from 8 o'clock the night before and were in bed. In each case three tests were performed. In the first test only adrenaline was given (test A). In the second and third tests, the test subjects were given antihistaminics before adrenaline injection, either chlortrimeton (test C) or thephorin (test T), the investigator not knowing which of these substances had been given. With 7 of the test subjects »test C» was performed second, and in 8 cases »test T» came the second. Between the first and second tests there was an interval of 2—8 days (av. 6.3) and between the second and third tests one of 4—15 (av. 7.1).

Adrenaline (l-adrenaline hydrochloride-»Exadrin» Astra) was

given intramuscularly into the deltoides muscle, the dose being 0.5—1.0 mg (average 0.66 mg). The dose per kiloweight was 0.007—0.013 mg (average 0.01). In one case where the adrenaline dose was 0.5 mg an extra dose of 0.4 mg was given twenty minutes later. 8 mg of chlor-trimeton was given twice *per os*. The first dose was given at 22 hrs. the evening before, the second in the morning about two hours before adrenaline injection. Altogether three tablets of thephorin (25 mg each) were given, the first dose being at 16 hrs. the day before the test. Otherwise the dosing was the same as with chlor-trimeton. If necessary the test subjects got a short acting sedative N-methylcyclohexenylmethylbarbituric acid before going to sleep.

The pulse was counted from the radial artery and the blood pressure measured with a Riva-Rocci apparatus. Determinations took place immediately before the adrenaline injection and 5—10—15—30—45—60—90—120 minutes after. In some cases it proved to be necessary to make determinations between the above-mentioned times also. The blood sugar was determined (according to Hagedorn-Jensen). immediately before adrenaline injection and 15—30—45—60—75—90—120 minutes after from a specimen taken from the top of the finger.

RESULTS

The average initial values of the blood pressure, pulse, and blood sugar in the different tests can be seen in Table 1. Table 2 shows the changes from the initial value of circulation and blood sugar in special cases at the time when the effect of the drugs was at its strongest.

In Fig. 1 are set out the average curves of the systolic, diastolic and pulse pressure changes during the tests reckoned as percentages of the initial value. Figure 2 shows the average curves of the pulse and Figure 3 of the blood sugar in different tests. In six cases (5 during »test T» and 1 during »test C») the maximum value of the systolic pressure, pulse pressure or pulse was obtained at points which are not observed in the drawing up of the average curves.

When comparing the reaction of the same person in different tests it was found that the systolic and pulse pressure rose most strongly from its initial value in 11 cases in »test T» and in 2 cases

TABLE 1

THE INITIAL MEAN VALUES OF CIRCULATION AND BLOOD SUGAR (CASE NUMBER 15) AFTER PREMEDICATION WITH CHLOR-TRIMETON (TEST C), THEPHORIN (TEST T) AND IN CONTROLS (TEST A).

Test	A	C	T
Systolic pressure (mm. Hg)	125	121	124
Diastolic pressure (mm. Hg)	79	80	81
Pulse pressure (mm. Hg)	46	41	43
Pulse rate per minute	69	68	72
Blood sugar (mg. %)	82	83	78

TABLE 2

CIRCULATORY AND BLOOD SUGAR CHANGES (CASE NUMBER 15) AFTER ADMINISTRATION OF ADRENALINE (TEST A), CHLOR-TRIMETON + ADRENALINE (TEST C) AND THEPHORIN + ADRENALINE (TEST T)

	Test	A	C	T
Systolic Pressure	Maximum rise (mm. Hg)	40	40	85
	Rise $\geqq$ 40 mm. Hg. Case number	1	3	9
	No rise. Case number	2	0	0
Diastolic Pressure	Maximum fall (mm. Hg)	25	25	35
	Fall $\geqq$ 20 mm. Hg. Case number	5	4	5
	No fall. Case number	0	0	0
Pulse Pressure	Maximum rise (mm. Hg)	50	50	80
	Rise $\geqq$ 50 mm. Hg. Case number	1	1	8
	No rise. Case number	0	0	0
Pulse Rate	Maximum rise per min.	26	26	40
	Rise $\geqq$ 20 per min. Case number	5	7	11
	No rise. Case number	0	1	0
Blood Sugar	Maximum rise (mg. %)	77	79	78
	Rise $\geqq$ 50 mg. %. Case number	3	5	9
	No rise. Case number	2	0	1

in »test C». In two cases the rise was equally big in »tests C and T». The diastolic pressure decreased most noticeably in 5 cases during »test T», in three cases in »test A» and in two cases in »test C». In other cases the same minimum value was obtained in several tests. The pulse rose most from its initial value in 11 cases in »test T», in

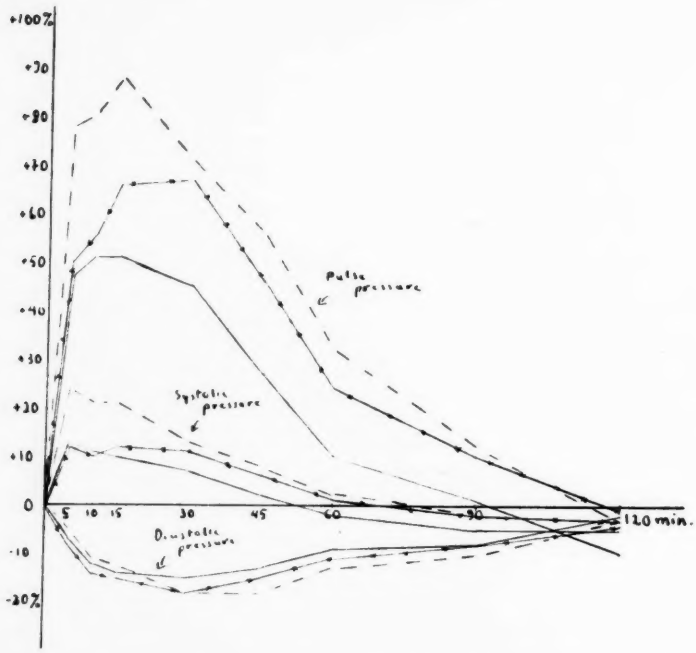

Fig. 1 — The average systolic, diastolic and pulse pressure curves in antihistamine-adrenaline tests calculated as percentages of the mean initial value. ——— after giving adrenaline, ×—×—× after chlor-trimeton and adrenaline, - - - - - - after thephorin and adrenaline.

2 cases in »test A» and in 1 case the rise was as big in »tests C and T». The blood sugar increased most strongly in 8 cases in »test T» in 5 cases in »test C» and in 2 cases in »test A».

Noticeable objective side effects came out in 4 cases during »test T». In these cases extra-systole and irregularities in the working of the heart were found. In two cases pulse deficite appeared and in one of them bigeminia (Figure 4).

13 test subjects reported that the subjective effect was strongest during »test T», in one case the effect was felt strongest in »test A»

and in one case equally strong during »tests A and C». 5 test subjects reported the subjectively weakest effect to be in »test C», in two cases the effect felt weakest was in »test A» and similary in two cases in »test T». The rest considered the effect of »tests A and C» equally weak.

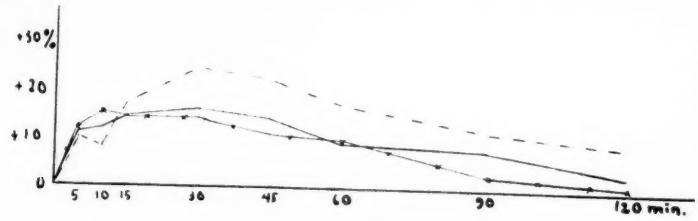

Fig. 2. — The average pulse curves in antihistamine-adrenaline tests calculated as percentages of the mean initial values. ——— after giving adrenaline, ×—×—× after chlor-trimeton and adrenaline, - - - - - after thephorin and adrenaline.

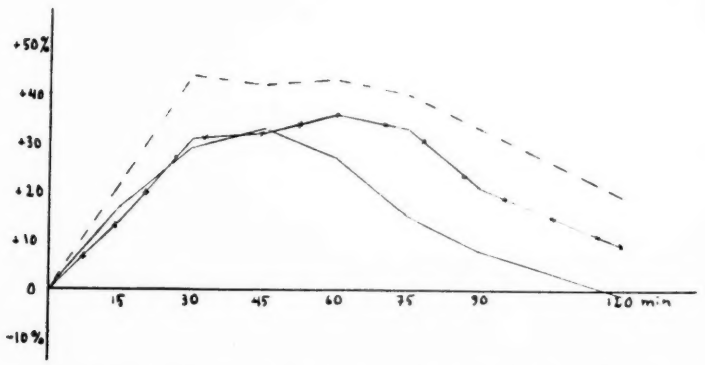

Fig. 3. — The average blood sugar curves in antihistamine-adrenaline tests calculated as percentages of the mean initial values. ——— after giving adrenaline, ×—×—× after chlor-trimeton and adrenaline, - - - - - after thephorin and adrenaline.

DISCUSSION

In this respect the result was unexpected — that thephorin, which contrary to chlor-trimeton, blocked the effect of adrenaline in blood pressure tests on animals, in this case strengthened it. The antihistamine doses used were in both cases considered normal (7). Antihistaminics do not have effect in therapeutic doses upon the

normal circulation (6, 19). In our experiments also the blood pressure and the heart rate before adrenaline did not differ from that of the control group. Antihistaminics also do not change the fasting blood sugar or only to a small extent in large doses in the test animals (16, 27). Scheidhauer (23) got with antistine *per os*

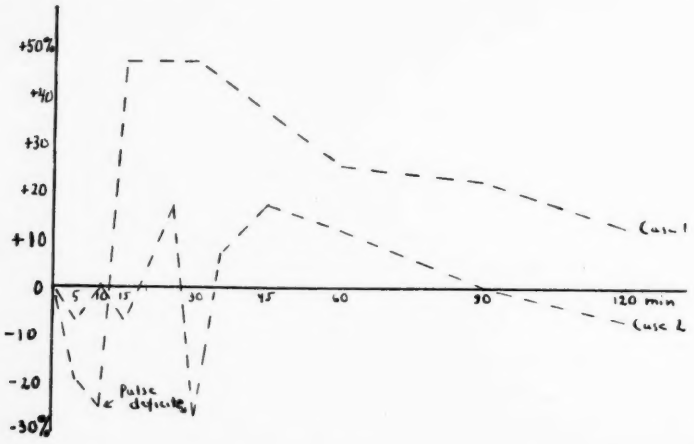

Fig. 4. — The pulse curves of two test subjects calculated as percentages of the initial values after giving thephorin and adrenaline. In both cases the initial adrenaline dose was 0.5 mg, but in case 2 an extra dose of 0.4 mg was given at the 20. min. point.

in children a blood sugar rise of 20—35 mg %, but this could be a result of the central irritating effect of the antihistaminics on the growing individual. Excitation is met with in children much more often than in adults after therapeutic doses (7). In our experiments the average initial value of the blood sugar did not differ from that of the control group. After thephorin the hyperglycemia response to adrenaline was increased in height and duration. It seemed also that chlor-trimeton lengthened the response though not so clearly. Here there is a discrepancy from the abovementioned animal tests. The adrenolytic effect of thephorin upon the isolated organs, such as the seminal vesicle of guinea-pig, uterus and periferal circulation as judged from rats mesoappendix or perfused legs or rabbit ear is one of the strongest antihistaminics (9, 10, 17, 20). The adrenolytic effect of chlortrimeton, and near to it trimeton, on these objects is one of the weakest of the antihistaminics (8—10, 20). On the

other hand, it must be remembered that in blood pressure tests anthisan for example as a dose of 15 mg/kg has adrenolytic potency, but as a dose of 5—7 mg/kg adrenomimetic potency (22).

Typical of all antihistaminics is their ability to block the effect of acetylcholine as well as vagus irritation (9, 10). Loew *et al.* (18) consider the fact that benadryl increases the adrenaline blood pressure response in dogs under anesthesia to be dependent upon its atropine properties. However the fact that after injecting a large amount of atropine, benadryl still increases the adrenaline response (5), speaks against the result of this test.

Thephorin is the only antihistamine substance whose side actions are not sedative but merely exciting (2, 3). This would support the view that in the human being thephorin has effect on the adrenaline reaction mainly in a central way. Chlor-trimeton is lacking in stimulating properties and its sedative action is also very slight (3, 31).

The results indicate that care must be used when giving adrenaline to a patient during thephorin medication. It cannot be said if the potentiating ability of thephorin upon adrenaline effect comes forth also in relaxing the bronchial spasm. In anaphylactic shock of guinea-pigs this could not be shown (20). It may be mentioned that ephedrine also, when used with pyribenzamine and thephorin has been said to be needed in smaller quantity in preventing asthmatic attacs than when without antihistamine treatment (14). Pyribenzamine and adrenaline also potentiate each other in preventing bronchial asthma (15).

SUMMARY

0.5—1.0 mg of adrenaline hydrochloride was injected intramusculary into 15 test subjects and the pulse rate, blood pressure and blood sugar was observed. The same test was carried out after premedication with chlor-trimeton and thephorin. After thephorin, the adrenaline response strengthened, especially the systolic blood pressure and pulse pressure. The fall of diastolic pressure, the increase of the pulse rate and the blood sugar were not enhanced so clearly. The subjective symptoms after thephorin were also increased. After chlor-trimeton there was no difference from the control group or only a slight increase in the responses.

REFERENCES

1. Chen, G., and Clarke, I. G.: J. Pharmacol. & Exper. Therap. 1948: *93:*175.
2. Cohen, M. B., Davis, H. B., and Mowry, W. A.: Am. J. Med. 1948: *5:*44.
3. Criep, L. H., and Aaron, T. H.: J. Allergy 1948:*19:*304.
4. D'Angostino, L.: Boll. Soc. ital. biol. sper. 1947:*23:*958.
5. Fabinyi-Szebehely, M., and Szebehely, J.: Acta pharmacol. et toxicol. 1952:*8:*343.
6. Feinberg, S. M.: J.A.M.A. 1946:*132:*702.
7. Feinberg, S. M., Malkiel, S., and Feinberg, A. R.: The Antihistamines. The Year Book Publishers, Chicago 1950.
8. Fleckenstein, A.: Brit. J. Pharmacol. 1952:*7:*553.
9. Haas, H.: Histamin und Antihistamine I. Editio Cantor, Würtenberg 1951.
10. Haas, H.: Histamin und Antihistamine II. Editio Cantor, Würtenberg 1952.
11. Halpern, B. N., Cruchaud, S., Vermeil, G., and Roux, J. L.: Arch. internat. Pharmacodyn. 1950:*82:*425.
12. Halpern, B. N., Hamburger, J., and Cruchaud, S.: Compt. rend. Soc. biol. 1947:*141:*1038.
13. Halpern, B. N., Hamburger, J., and Cruchaud, S.: Acta Allergol. 1948:*1:*97.
14. Kallós, P., and Kallós-Deffner, L.: Internat. Arch. Allergy & Appl. Immunol. 1951:*1:*189.
15. Koepf, G. F., Arbesman, C. E., and Lenzner, A.: Fed. Proc. 1946: *5:*56.
16. Komrad, E. L., and Loew, E. R.: J. Pharmacol. & Exper. Therap. 1951:*103:*115.
17. Lehmann, G., Randall, L. O., and Hagan, E.: Arch. internat. Pharmacodyn. 1949:*78:*253.
18. Loew, E. R., MacMillan, R., and Kaiser, M. E.: J. Pharmacol. & Exper. Therap. 1946:*86:*229.
19. Mc Gavack, T. H., Elias, H., and Boyd, L. J.: J. Lab. & Clin. Med. 1946:*31:*560.
20. Paasonen, M. K.: Ann. med. exper. et biol. Fenniae. 1953:*31:*Suppl. 7.
21. Reuse, J. J.: Compt. rend. Soc. Biol. 1948:*142:*638.
22. Reuse, J. J.: Ann. Soc. Royale Sc. Méd. et Natur. de Bruxelles 1949: *2:*93.
23. Scheidhauer, E.: Monatsschr. Kinderh. 1950:*98:*262.
24. Schmiterlöw, C. G., and Wessman, C. G.: Acta physiol. scandinav. 1951:*23:*31.
25. Stone, C. A., and Loew, E. R.: Fed. Proc. 1949:*8:*335.
26. Susina, S. V., and Unna, K. R.: J. Pharmacol. & Exper. Therap. 1951:*101:*34.

27. Tepperman, J., Rakieten, N., Birnie, J. H., and Diermeier, H. F.: J. Pharmacol. & Exper. Therap. 1951:*101:*144.
28. Tislow, R., La Belle, A., Makowsky, A. J., Reed, M. A. G., Cunningham, M. D., Emele, J. F., Grandage, A., and Roggen hofer, R. J. M.: Fed. Proc. 1949:*8:*338.
29. Vermeil, G., Halpern, B. N., and Cruchaud, S.: Compt. rend. Soc. biol. 1949:*143:*77.
30. Winter, C. A.: Proc. Soc. Exper. Biol. & Med. 1949:*72:*122.
31. Ziporyn, M.: Med. Times 1950:*78:*205.

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI, AND THE INSTITUTE OF TECHNOLOGY, HELSINKI

STUDIES OF THE GERMICIDAL ACTION OF SOME RARE EARTH-METALS

by

A. P. MUROMA

(Received for publication October 16, 1953)

Scandium, yttrium and 15 lanthanids, located below aluminium in group IIIa of the periodic table of elements, are called rare earth-metals. Literature on the toxicity of metals generally mentions only the most common, viz., cerium, the toxicity of which is somewhat higher than that of aluminum according to some sources, according to others somewhat lower (2, 4, 6).

The object of the present paper is to study the relative germicidal action of 8 cations belonging to the group of rare earth-metals. As a check, the germicidal action of copper, barium and aluminum was studied. The rare earth-metals used for the work have been isolated by Professor O. Erämetsä, Institute of Technology; their purity is in excess of 99 per cent. The cations examined and their atomic weights are listed below:

Rare Earth-metals		*Other Metals*	
Yttrium	Y — 89	Cu — 64	
Lanthanum	La — 139	Ba — 137	
Cerium	Ce — 140	Al — 27	
Praseodymium	Pr — 141		
Neodymium	Nd — 144		
Samarium	Sm — 150		
Europium	Eu — 152		
Ytterbium	Yb — 173		

With the exception of $CuSO_4$ and $CeNO_3$ the salts of hydrochloric acid of the metals were used. It has been shown that the toxicity of salts of simple inorganic acids such as hydrochloric acid, sulphuric acid and nitric acid, is principally attributable to the cation, the anion being relatively indifferent (1, 3, 5).

The germicidal action of the salts was examined against four bacteria species as test organisms, viz., Salmonella typhi murium, Escherichia coli, Staphylococcus aureus and Streptococcus haemolyticus. No germistatic tests were made by mixing the metal salt in the culture medium. This was partly because of the short supply of the quantities of metals available and partly because these metals react fairly strongly with ordinary culture media, causing precipitation.

METHOD AND RESULTS

10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} normal solutions were made of the metal salts and 0.9 ml of each solution was pipetted into the test tubes. The bacterial suspension, grown for 24 hours in broth, was diluted with water 1:100 and 0.04 ml of the dilution was added to each test tube. The tubes were shaken carefully. The test tubes

TABLE 1

THE TOXICITY VALUE. EFFECT OF $CUSO_4$ ON SALMONELLA TYPHI MURIUM

	The Concns of $CuSO_4$				Toxicity value
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
5 min.....	+	+	+	+	0
60 min.....	—	—	—	+	3
24 hr.	—	—	—	—	4

were kept at room temperature, and after an incubation period of 5 minutes, 60 minutes and 24 hours sub-cultures were taken from them on Drigalski-Conradi agar with a platinium wire, loop diameter 4 mm. The results were given as toxicity values indicating the number of the metal concentrations whose sub-cultures showed no growth. Table 1 illustrates an example of the assessment of the toxicity value. Each test were carried out twice. If the figures showed a discrepancy their mean was taken. The results are given in table 2.

TABLE 2

RESULTS OF THE GERMICIDAL TESTS

Toxicity value	Salmonella typhi murium			Escherichia coli		
	5 min. Exposure	60 min. Exposure	24 hr. Exposure	5 min. Exposure	60 min. Exposure	24 hr. Exposure
0	La Ce Cu Ba Al Y	La Al Ba	Ba	La Ce Pr Nd Sm Eu Cu Ba Al Y	La Ce Pr Nd Sm Eu Ba Al Y	La Pr Ba
0.5						Ce
1				Yb		Al
1.5						
2	Pr	Ce				Nd Sm
2.5		Pr			Cu	Eu
3	Nd Sm Eu	Nd Sm Eu Cu	Al			Y
3.5	Yb		Ce Pr Nd Eu			
4		Yb Y	La Sm Yb Cu Y		Yb	Yb Cu

Toxicity value	Staphylococcus aureus			Streptococcus haemolyticus		
	5 min. Exposure	60 min. Exposure	24 hr. Exposure	5 min. Exposure	60 min. Exposure	24 hr. Exposure
0	La Ce Pr Nd Sm Eu Yb Cu Ba Al Y	La Ce Pr Nd Sm Eu Ba Al	La Ce Ba Al	La Ce Pr Nd Sm Eu Yb Cu Ba Al Y	La Ce Pr Nd Sm Eu Yb Ba Al Y	La Ce Pr Ba Al
0.5		Y	Pr			
1		Yb	Y		Cu	Yb Y
1.5			Nd			Eu
2			Eu			
2.5						Nd
3		Cu	Sm			Sm
3.5			Yb			
4			Cu			Cu

DISCUSSION

Klem has compiled a table in which the lanthanid group and barium and hafnium are arranged by increasing atomic weights with the metals easiest to reduce on the left and those most difficult to reduce on the right:

Ba	*La*	*Ce*	*Pr*	*Nd*	Pm	*Sm*	The metals investigated are
Eu	Gd	To	Dy	Ho	Er	Tm	given in italics in the table.
Yb	Cp	Hf					

The general rule that the toxicity of metals for germs increases with their atomic weight is applicable on the whole to the metals in the above table. Thus Ba<Eu<Yb, and if we compare the relative toxicity of metals from the left to the right it becomes clear that in the upper row it increases almost without exception. Yttrium, which has been placed in the periodic table above the group of lanthanids, proved, despite its relatively small atomic weight, to be more toxic than most of the lanthanids examined. On the other hand the toxicity of aluminum, placed above yttrium, proved relatively small. With the exception of ytterbium, copper is more toxic than the rare earth-metals studied.

When a few test series were performed with 10^{-1} normal metal salt solutions they were found in some cases to have a weaker germicidal effect than milder concentrations. No regularity was established here, which can be attributed, at least in part, to test errors.

SUMMARY

The relative germicidal action of salts of 8 rare earth-metals, Y, La, Ce, Pr, Nd, Sm, Eu, and Yb, was examined against four bacteria species as test organisms. In addition, Cu, Ba and Al were investigated as a check. It was found that the toxicity of the rare earth-metals examined increases on the whole with their atomic weights. An exception was Y which despite its relatively small atomic weight was more toxic than several of the rare earth-metals investigated. Compared with them the toxicity of Al and Ba proved to be relatively low. With the exception of ytterbium, copper was more toxic than the rare earth-metals examined.

REFERENCES

1. Chick, H.: J. Hyg. 1908:*8:*92.
2. Eisenberg, Ph.: Cbl. Bakt. 1919:*82:*69.
3. Holm, G. E., and Sherman, J. M.: J. Bakt. 1921:*6:*511.
4. Winslow, C.-E. A., and Hotchkiss, M.: Proc. Soc. Exp. Biol. 1922:*19:* 314.
5. Andresen, P. H.: Cbl. Bakt. 1928:*105:*444.
6. Wilson, G. S., and Miles, A. A.: Topley and Wilsons Principles of Bacteriology and Immunity. London. 1946.
7. Koulumies, R.: Acta path. et microbiol. scand. Suppl. LXIV. 1946.
8. Alha, A. R.: Acta path. et microbiol. scand. Suppl. LXV. 1946.

FROM THE INSTITUTE OF PHYSIOLOGY, UNIVERSITY OF HELSINKI

BILIRUBIN ELIMINATION FROM BLOOD DURING ACUTE HYPOXIA

by

E. JALAVISTO, H. LYBECK, H. A. SALMI, and I. SUNDHOLM

(Received for publication October 23, 1953)

In a previous paper (5) the elimination of bromsulphalein from blood during hypoxia was studied. No evidence of impairment of the function responsible for elimination of BSP from the blood was seen in dogs breathing oxygen-nitrogen mixtures of 7—8 per cent O_2.

Since according to Rich (8) the rate of elimination of bilirubin is decreased in anoxic conditions and since certain observations with chronic exposure to high altitude point in the direction of bilirubinaemia as a result of liver impairment caused by the low atmospheric oxygen pressure (7) experiments of elimination of exogenous bilirubin during acute hypoxia, were performed. It has been claimed by Harrop and Barron (3) that this function test is more sensitive than e.g. the bromsulphalein test. It was thought that if acute hypoxia is induced it might bring a latent insufficiency of the liver excretory function manifest and would thereby increase the sensitivity of the bilirubin excretion test, and possibly have practical value when applied to conditions with subclinical impairment of liver function. The experimets might on the other hand give some indications of the susceptibility of the liver for lack of oxygen. It is a quite common belief that the liver is sensitive against lack of oxygen, yet the evidence is mostly derived from pathological anatomical studies after impairment of circulation of the liver or

after excessive exposure to low oxygen pressure (4). Less severe anoxia, i.e. reduction of the oxygen pressure in the inspiratory air corresponding to 7—8 per cent 0_2 was, however, found to give inconclusive evidence of disturbance of liver excretory function in anaesthetized rats, whereas breathing of 6 % oxygen clearly decreased the bilirubin output in the bile (2). Tanturi and Ivy (9) came to similar results when studying the volume output of bile during hypoxia of short duration. They found hardly any decrease of the volume flow of bile in anaesthetized dogs breathing mixtures with 7.5—10 per cent oxygen during 20 minutes. These results are rather an indication of the relative resistance of the liver against lack of oxygen than of its susceptibility. Many other excretory functions, e.g. gastric and pancreatic secretion are namely definitely decreased already at higher oxygen tensions i.e. at tensions corresponding to 8—10 per cent of oxygen in inspired air. In Hanzon's experiments no systematic study of the critical level of oxygen deficiency affecting the excretory functions of the liver was undertaken. In the present study this limit was tried to establish.

THE PRESENT INVESTIGATION

The experiments were performed on 5 mongrel dogs, 3 of which were females, all about 1—1.5 years of age. Four kinds of experiments were made

1) normal control experiments,
2) hypoxic in conscious state,
3) normal under nembutal anaesthesia,
4) hypoxic under nembutal anaesthesia.

In the experiments bilirubin (Eastman Kodak Co or Hoffmann la Roche) solved in 2.5 per cent boiling sodium carbonate and filtered through sterile filter paper was injected as 0.5 per cent solution in an amount corresponding approximately to a dose of 1.7—2.5 mg/kg body weight. After 3 min. a blood sample amounting to 5—8 ml from a vein not used for injection of bilirubin was taken and analyzed for bilirubin. Further samples were thereafter taken 18, 33, 63, 93, 123, 153 and 183 minutes after the injection of bilirubin. The blood was taken in centrifuge tubes shielded from

light with black paper convers. The determinations of bilirubin concentration were made only from clear serum samples showing no hemolysis. When possible double determinations were made. The arithmetic mean of deviations in parallel determinations was 0.06, the number of determinations 85 × 2. In ten instances the difference exceeded 0.1 mg % of bilirubin. The samples were analyzed photometrically according to the method of K. Jendrassik and R. A. Cleghorn (6) with a »Lumetron» electrophotometer. Instead of the light filters of 530 and 430 $\mu\mu$ wave lengths used in the original method the filters 550 and 440 $\mu\mu$ were used. This seemed justified in view of the fact that no difference in bilirubin absorption between these wave lengths was found in control determinations performed with a Beckman spectrophotometer. The method of Jendrassik and Cleghorn was chosen instead of simple colorimetry, because in hypoxia the colour of the blood serum might change.

In the hypoxic experiments the oxygen nitrogen mixture was delivered from a gas container through a normal type of oxygen inhalator to a gas mask, which the dogs carried without much protestation. The dogs were standing at a Pavlow stand. The hypoxic gas mixture was inhaled during the entire experimental period of three hours. A longer duration of the experiments was not considered necessary. Besides since no low pressure chamber was at our disposal it would have been hardly feasible to extend the period of gas delivery beyond three hours. In order to be sure that no air was sucked in through the rubber ring of the gas mask around the nose of the dog the gas mixture was let in with slight positive pressure. The experiments in which the dogs were anaesthetized (2 ml per 1 kg body weight Abbot veterinary Nembutal) were similar to the hypoxia experiments in conscious dogs, with the exception that the degree of hypoxia was controlled by blood samples taken from the femoral artery and analyzed for their oxygen content according to the modification of Yiengst (10) of the v. Slyke and Neill manometric method. The oxygen carrying capacity was calculated from the content of electrophotometrically determined hemoglobin in alkaline solution by multiplying the amount of hemoglobin in g with 1.32 instead of the theoretical 1.34, since part of the photoelectrically determined oxygen is not available for the oxygen transport.

TABLE 1

Date 1953	Dog	Experiment	Bilirubin Concentration in 3 min. Sample = 100 per Cent mg %	Bilirubin per Cent of 3 min Value	
				in 18 min. sample	in 33 min. sample
15/5	Aspasia 10 kg	Hypoxia	2.84	42.5	24
23/4	Viiru 15 kg	»	2.58	43	33
28/5	Vena 11 kg	»	2.57	35.5	28
29/4	Keiju 10 kg	»	2.42	38	22
14/4	Ph 5.5 kg	Control	2.29	34.5	15
31/3	Aspasia	»	2.12	30	31
16/4	Kimmo 14 kg	»	2.07	49	34
21/4	Aspasia	»	2.00	34	30
6/5	Kimmo	Hypoxia	1.91	43.5	39.5
18/4	Vena	Control	1.76	35	27.5
13/3	Ph	»	1.60	42	19
6/3	Vena	»	1.46	45	39
17/3	Kimmo	»	0.98	55	36
28/3	Viiru	»	0.93	59	27.5
3/3	Viiru	»	0.93	46	24
4/5	Ph	Hypoxia	0.87	68	52
19/5	Viiru	»	0.62	48	40

RESULTS

The initial rate of bilirubin elimination from blood was found to be dependent upon the concentration of bilirubin in blood. Semilogarithmic or percentage curves of the elimination are therefore misleading. When hypoxic and normal elimination curves are compared, it is therefore necessary to see that the 3 min. concentration values do not much differ. If viz. the bilirubin content in the 18 min. sample is expressed as percentage of the 3 min. value, the highest percentages (slowest elimination) are encountered in the cases with the lowest blood bilirubin concentration. Table 1, in which the experiments are arranged according to the initial (post injection) blood bilirubin concentration shows clearly this negative correlation between blood bilirubin level and rate of elimination. The rank correlation coefficient according to Spearman gives a value $-0.74 \pm 0.10 : n = 21; P < 0.0027$. The corresponding value

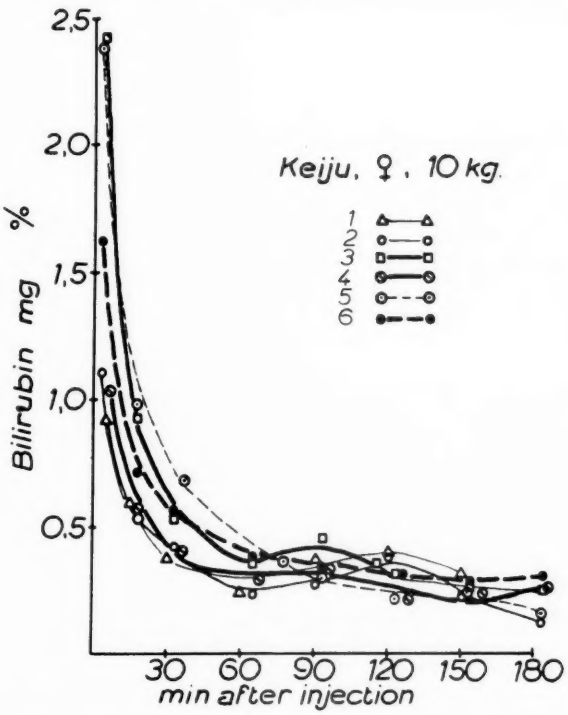

Fig. 1. — Dog Keiju, ♀. 1) 21. 3. 53. Control experiment, conscious state. 2) 11. 4. 53. Control experiment, conscious state. 3) 29. 4. 53. Hypoxia (6.5 % O_2), conscious state. 4) 3. 6. 53. Hypoxia (7 % O_2), conscious state. 5) 14. 8. 53. Control experiment, nembutal anaesthesia. 6) 4. 8. 53. Hypoxia (8 % O_2), nembutal anaesthesia.

for the 33 min. sample is however -0.20 ± 0.21, which shows that this value seems not to be significantly influenced by the initial level of blood bilirubin concentration.

In conscious dogs 5 experiments with breathing of 6.5—8 per cent oxygen were made. None of the bilirubin elimination curves showed any significant difference as compared to the normal elimination curves (Fig. 1—5).

In experiments with narcotized animals the detoxication of the barbiturate might mean a stress layed upon the liver cells and increase their oxygen requirements. Dragstedt and Mills (1) e.g. have found a definite prolongation of the time for complete removal of bilirubin from blood i dogs anaesthetized with ether plus barbital sodium. Since experiments in which the liver function has been

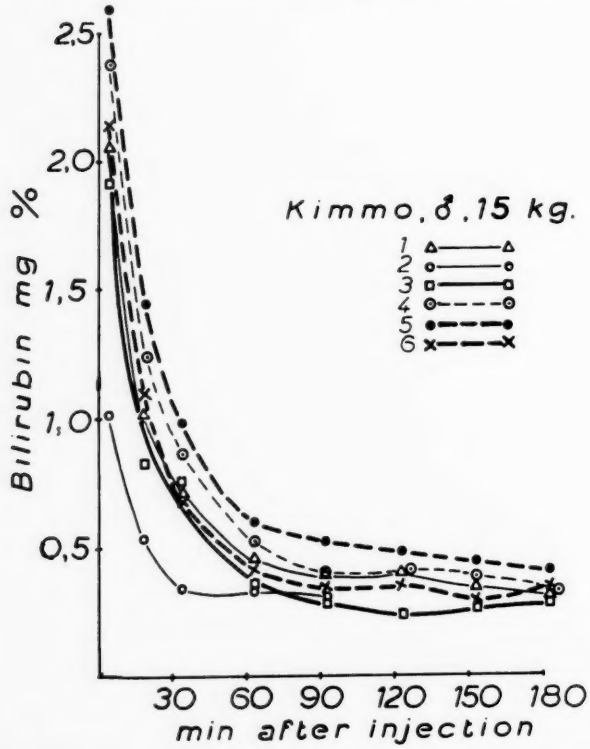

Fig. 2. — Dog Kimmo, ♂. 1) 16. 4. 53. Control experiment, conscious state. 2) 17. 3. 53. Control experiment, conscious state. 3) 6. 5. 53. Hypoxia (8 % O_2) conscious state. 4) 10. 9. 53. Control experiment, nembutal anaesthesia. 5) 22. 6. 53. Hypoxia (7 % O_2), nembutal anaesthesia. 6) 6. 8. 53. Hypoxia (8 % O_2), nembutal anaesthesia.

impaired by hypoxia (2) have been made with anaesthetized animals, it was thought necessary to perform experiments with anaesthetized dogs as well. In three experiments with 8—9 per cent oxygen mixture leading to an arterial saturation of oxygen of 55, 56 and 62 per cent no difference from normal elimination could be observed. In one experiment with only 49 per cent saturation in an experiment with inhalation of 7 per cent oxygen mixture a slight slowing down of bilirubin excretion was seen, but at the end of the three hours period, practically no greater retention than normally was present. (Fig. 2: No 5.) If the bilirubin concentration in the 3 min. samples exceeded 2 mg per cent, as a rule, the initial bilirubin level was not reached in three hours. However, no clearcut

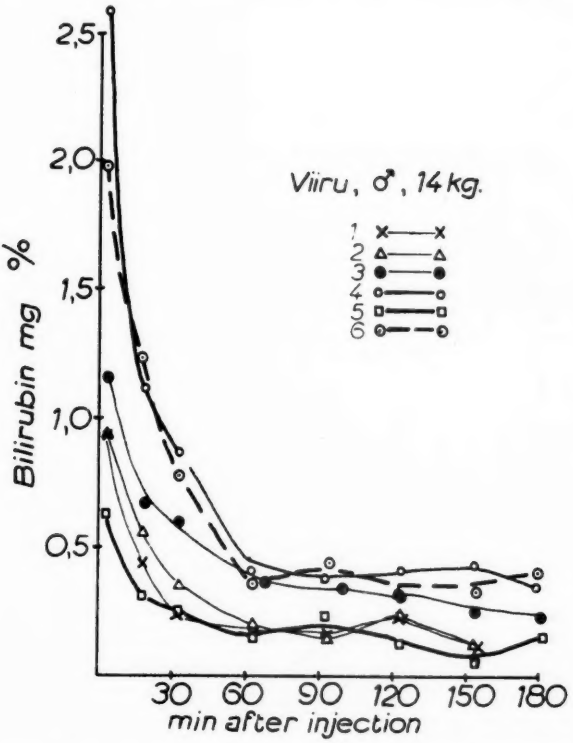

Fig. 3. — Dog Viiru, ♂. 1) 1. 3. 53. Control experiment, conscious state. 2) 28. 3. 53. Control experiment, conscious state. 3) 8. 9. 53. Control experiment, conscious state. 4) 23. 4. 53. Hypoxia (during 33 min. 5.6 % O_2, thereafter 19 % O_2), conscious state. 5) 19. 5. 53. Hypoxia (8 % O_2), conscious state. 6) 11. 8. 53. Hypoxia (7 % O_2), nembutal anaesthesia.

difference between normal, hypoxic and anaesthetized animals could be noted. The elimination curve was in this part already practically horizontal.

On the whole the anaesthesia experiments seem to indicate that the elimination of bilirubin from the blood remains satisfactory even if the oxygen saturation of arterial blood is lowered to the extreme values of 49—55 per cent.

DISCUSSION

When the results of the present study are being interpreted, the same difficulties as those found in the interpretation of the BSP elimination test present themselves. The question whether the

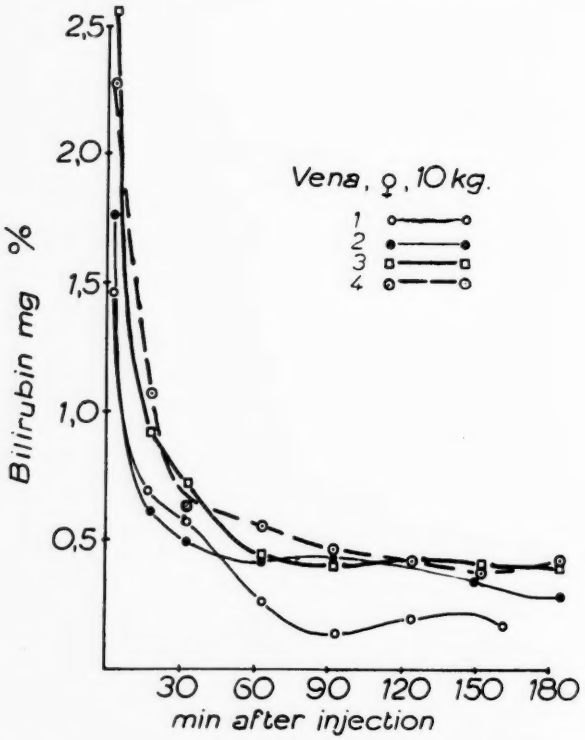

Fig. 4. — Dog Vena, ♀. 1) 6. 3. 53. Control experiment, conscious state. 2) 18. 4. 53. Control experiment, conscious state. 3) 28. 5. 53. Hypoxia (7.5 % O_2), conscious state. 4) 18. 8. 53. Hypoxia (9 % O_2), nembutal anaesthesia.

tolerance against lack of oxygen of the function responsible for bilirubin elimination is high because the reactions involved are not aerobic or require only a very moderate oxygen tension for their normal sequence cannot be satisfactorily settled. Furthermore, a vasomotor compensatory adjustment of the hepatic circulation during anoxia may provide a satisfactory supply of oxygen. Thirdly, the elimination of the dye from the blood may be a physical event ocurring in the cells of the RES and no information as to the *excretion* of bilirubin during the same degree of anoxia by the hepatic cells, are obtainable in experiments of the type of bilirubin load reported on above. The fact, however, that ligation of the bile ducts impair the elimination of bilirubin from blood (1) is much in favor of the view that the elimination is not simply a physical

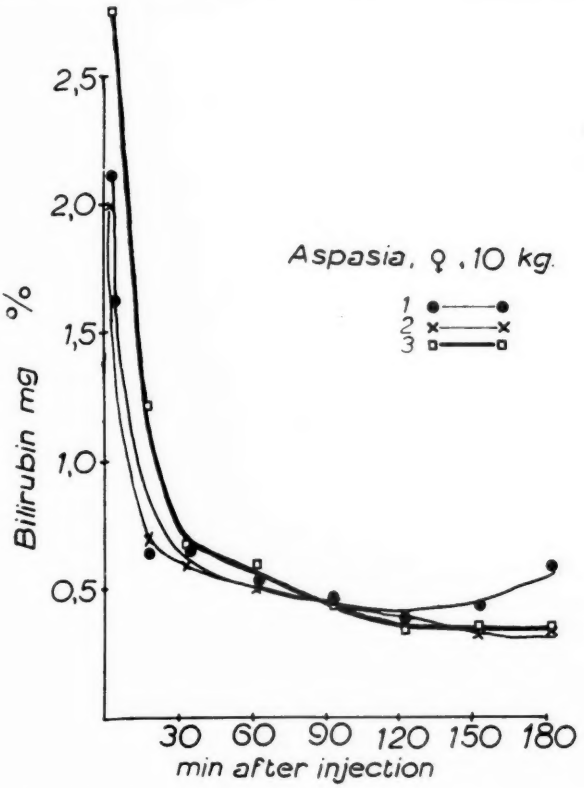

Fig. 5. — Dog Aspasia, ♀. 1) 31. 3. 53. Control experiment, conscious state. 2) 21. 4. 53. Control experiment, conscious state. 3) 15. 5. 53. Hypoxia (6.7 % O_2), conscious state.

absorption process but due to excretory function of the liver cells. Experiments with a continous infusion technique might give better information of the clearence of bilirubin by the secretory parenchymal cells but the high alkalinity necessary for solution of the dye prevents the use of such a technique. The only possibility of investigation would therefore be direct measurement of the bilirubin output in bile fistula dogs under otherwise equal conditions and equal degrees of hypoxia. If it could be shown that 7—8 per cent O_2 gas mixture clearly decreases the bilirubin excretion, it would be an indication of the fact that *blood bilirubin elimination tests are inadequate for testing the excretory function of the liver parenchyma.* On the other hand if the bilirubin output would show the same

tolerance against low oxygen pressure in arterial blood as the cells responsible for disappearence of the dye from blood the question of circulatory adjustment during hypoxia becomes increasingly interesting.

Experiments with the purpose of elucidating the circulatory adjustments affecting the oxygen supply of the liver during hypoxia, will be reported in a subsequent paper.

SUMMARY

1) Elimination of exogenous bilirubin in 5 normal, normal hypoxic, anaesthetized and anaesthetized hypoxic dogs was studied.

2) It was shown that the elimination of bilirubin was not impaired during inhalation of a 7—8—9 per cent oxygen nitrogen mixture with arterial oxygen saturations of only 49—56—62 per cent neither in conscious nor in anaesthetized dogs.

REFERENCES

1) Dragstedt, C. A., and M. A. Mills: Am. J. Physiol. 1937:*119:*713.
2) Hanzon, V.: Acta physiol. scand. 1952:*28:*Suppl. 101 pp. 1—268.
3) Harrop, G. A., and E. S. Y. Barron: J. Clin. Invest. 1931:*9:*577—87.
4) Himworth, H. P.: The Liver and its Diseases, Oxford, Blackwell, 1950.
5) Jalavisto, E., and H. Lybeck: Ann. med exp. & biol. Fenn. 1953: *31:*95—102.
6) Jendrassik, L., and R. A. Cleghorn: Biochem. Z. 1937:*289:*1.
7) Merino, C. F.: Blood. 1950:*5:*1—31.
8) Rich, A. R.: Bull. J. Hopkins Hosp. 1930:*47:*338.
9) Tanturi, C. A., and A. C. Ivy: Am. J. Physiol. 1938:*121:*61.
10) Yiengst, M. J.: Science 1950:*112:*205.

FROM THE INSTITUTE OF PHYSIOLOGY, UNIVERSITY OF HELSINKI, AND THE RESEARCH CENTER FOR THE AGED, SOCIETAS GERONTOLOGICA FENNICA

STANDING STEADINESS IN OLD AND YOUNG PERSONS

by

K. BOMAN and E. JALAVISTO

(Received for publication October 23, 1953)

The question whether it is possible to asess the biologic age of individuals, has during the last years received increasing interest. Murray (12) has to our knowledge been the first seriously to attack the problem and to test the value of various physiological experiments as indications of the age of the experimental subject. However, the number of tests among which the selection is to be made is rather limited. Most physiological function tests have not been correlated to the age of the subject. The coordinative functions of the central nervous system have received very little attention, yet from the gerontological point of view they represent one of the major problems. A survey of the theoretical background of determination of the biologic age has been given by one of us (6). The purpose of this contribution is simply to see whether a test indicating variations in the standing steadiness (»quantitative Romberg-test») introduced in physiological methodology by Miles (10) and as an optical recording method by Goldberg (4) has any value as a symptom of the aging of an individual. This test was chosen, among others, because unsteadiness of gait is one of the commonest signs and complaints in people who feel that they are growing old. Frucht (3) has also shown with a mechanical method of recording that the summated oscillations occurring when standing still increase with increasing age. Monjé (11) has studied the influence of hypoxia upon standing steadiness and noted an increase in the oscillations with decrease of the atmospheric pressure. Goldberg (4) applied the test

Fig. 1. — Camera stand with the subject.

for quantitative evaluation of disturbances in coordination after ingestion of ethanol and found it to be a rather sensitive test of intoxication. Standing steadiness has furthermore been shown to be independent of training (9) and it is not possible voluntarily to supress the oscillations. These last mentioned properties make the test well suited for repeted trials.

PRESENT INVESTIGATION

The experiments were made with young medical students in the age of 18—24 years and some members of the staff in the age of 25—30 years, in all 43 subjects. The group of old people consisted in 38 healthy inmates, 20 females and 18 males, of the home for aged in Käpylä and Oulunkylä, aged 61—88 years. In 20 out of the 38 old and in 14 out of the 43 young people weight discrimination experiments were also performed.

The method of Goldberg was modified, but the general idea was the same viz. that of photographing the movements of an

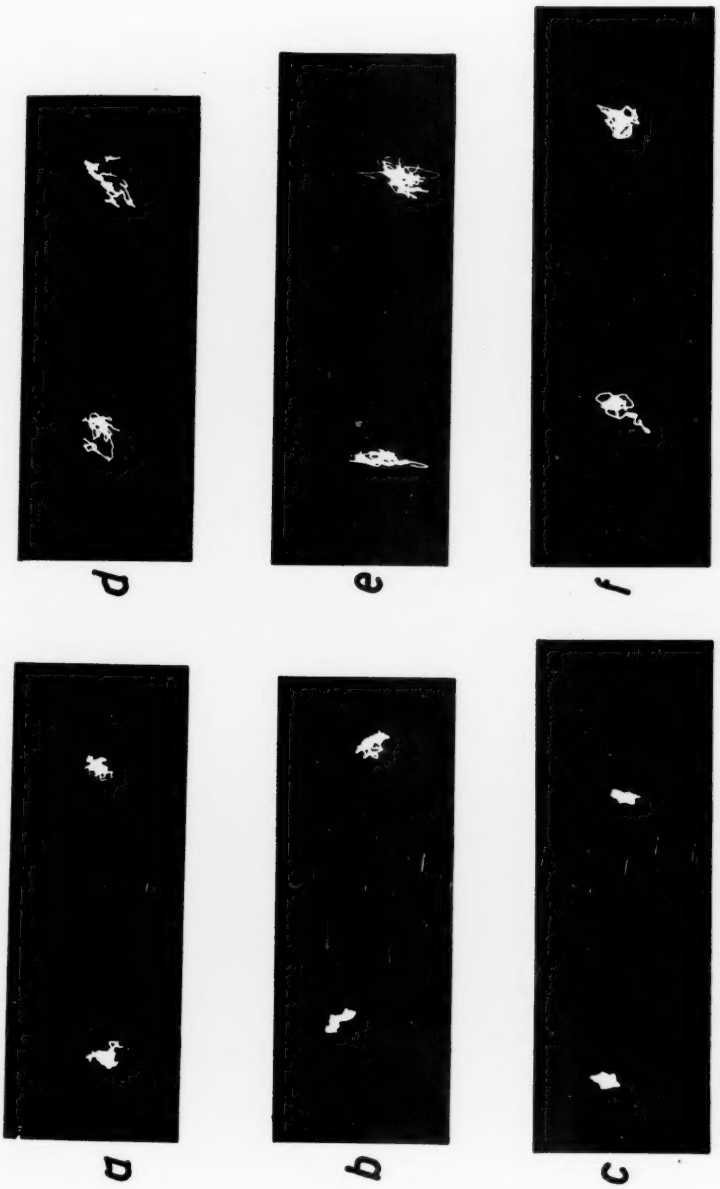

Fig. 2. — Contact reproductions of the photographed oscillations, a—c young subjects, d—f old subjects, double exposures of 1 minute duration each. (1 : 1).

TABLE 1

Age Years	Antero-posterior Swing		Lateral Swing		Area		n
I 18—30	41.7 ± 1.6		29.7 ± 1.8		78.3 ± 4.6 (n = 42)		43
II 62—80	56.4 ± 2.8		36.1 ± 2.7		98.6 ± 9.5		25
III ≥ 81	75.5 ± 6.3		49.5 ± 5.9		205 ± 30.5		13
Difference	Antero-posterior Swing	K	Lateral Swing	K	Area		K
III— I	33.8 ± 6.5	5.2	19.8 ± 6.2	3.2	127	± 30	4.2
III—II	19.1 ± 6.8	2.8	13.4 ± 6.7	2.0	106	± 32	3.3
II— I	14.7 ± 3.2	4.6	6.4 ± 3.2	2.0	20.3	± 10	2.0

$$K = \frac{\text{mean difference}}{\text{mean error of difference}}$$

illuminated spot fastened on the head of the experimental subject. A special stand for fastening of the photographic camera was constructed (Fig. 1). The position of the camera (Retina II/f: 2/50 mm + a front lense) could be adjusted according to the height of the subject, which could be read from a scale. The distance of the camera from the light spot on the head of the subject was exactly 30 cm. The light source consisted of an electric torch bulb covered with a metal box with a hole of only ½ mm in diameter and a silk paper fastened under it as to prevent scatter of the light. The recording was made in a darkened room the subject standing without boots the feet straight in the antero-posterior line. Double expositions of one minutes duration were made with only a short pause between the determinations. The exposed film was developed with a contrast developer. The picture of the light spot formed on the film an irregular pattern (fig. 2). This figure was magnified and drawn upon paper. The antero-posterior and the lateral diameter of the area of sway were measured. The excursions of the vertex were calculated in mm. Besides the area covered by the oscillations was measured planimetrically (table 1).

This procedure was chosen because the height of the subject does not influence the amplitude of swing. An artificial prolongation of the swinging radius as in the method Goldberg, who regulated the

distance between camera and light spot with help of an adjustable bar, fitted on the shoulders of the subject cannot therefore be considered as appropriate.

RESULTS

When the configuration of the area of swing is examined, it may be noted that in most instances i.e. in 53 out of 81 the lateral dimension is at least 10 mm smaller than the antero-posterior one. This holds true particularly for the old in which the oscillations are on the whole greater. Percentually, however, no great difference can be noted: in young the lateral diameter is on an average 71 per cent of the antero posterior diameter and in the old 65 per cent. Thus no principally different pattern of the oscillations exists in young and old people. Consequently the same method of measurement is applicable both for the young and the old.

If the first and second exposition are compared hardly any systematic difference is seen, the second antero-posterior diameter being on an average 4 mm smaller than the first one both in old and in young. The difference in the mean lateral diameter in the first and second exposition is even smaller 1.2 in the young and 1.5 mm in the old. The arithmetic mean of the deviation of the first and second determinations are in the young 10.0 mm in the antero-posterior and 6.8 mm in the lateral dimension. The corresponding values for the old are 18 and 13 mm respectively. Percentually the difference between first and second exposition is equally great both in the old and the young. The values given in the following refer to the mean of the two expositions.

The results are summarized in table 1. The series was divided in three age groups: I 18—30 years, II 62—80 and III 81 years and over. As seen from the table the mean amplitude of swing increases with increasing age. In the antero-posterior direction the means of group II differs statistically significantly both from the means of group I and that of group III. Curiously enough the difference of the means is as great (actually numerically greater) between the two groups with old people than between the young and group II. This fact seems to indicate that the impairment in the standing steadiness is a rather late manifestation of growing old, since in the group of 62—80 years old most values fall within the range of values

found in the young, and first beyond 80 years the amplitude of sway is uniformly increased. In figure 3 the frequency distribution of the individual values is seen. It may be noted that the extreme values belong to extreme age groups but that an overlapping of the values is considerable.

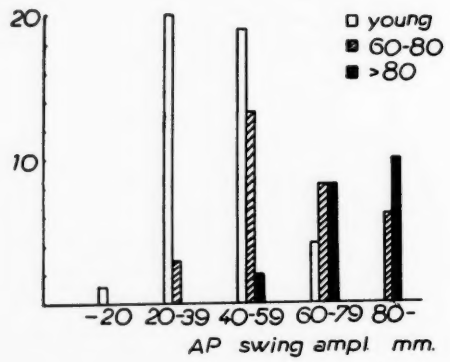

Fig. 3. — Distribution of values for the antero-posterior diameter of the area of swing in millimeters.

When the amplitudes of the lateral swing are compared the picture is much the same as for the antero-posterior swing. The mean amplitude increases with increasing age and the increase is most marked between the two groups of old people. However, the scatter of the values is relatively greater and the differences only probably significant the difference of two means of the age groups being only twice the mean error of the difference. The distribution of the values shows considerable overlapping of the values belonging to the different age groups (fig. 4). There is no difference in the amplitude of swing between the two sexes neither in the young nor in the old. The averages for young in the antero-posterior direction is viz. 41 for females 42 for males and in the old 62 mm and 64 mm respectively.

In order to make sure that no bias has been induced by the different height of the experimental subjects the correlation between height and amplitude of swing was examined among the young (group I) and the old (groups II and III). For sake of simplicity the Spearman rank correlation method was used. It could be shown that

there was no significant correlation neither in the group of young nor in those with old people the correlation coefficients being +0.03 in the young and +0.01 in the old.

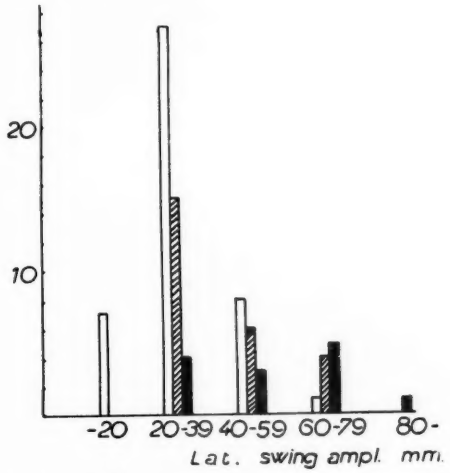

Fig. 4. — Distribution of values for the lateral diameter of the area of swing in millimeters, designations as in figure 3.

DISCUSSION

Several methods have been used for recording of the oscillations which occur during an attempt to stay still. The first graphic method (15) using a stray fastened on the head and a horisontally placed smoked glas plate on which the stray wrote the movements of the head, is not quite satisfactory (8) and particularly not very convenient. The device called »Ataximeter» (Miles, 10) by german investigators »Arbeitssamler» (Fick, 2) which records oscillations mechanically with help of two perpendicular strings, is not without objection because the frictional resistance and the mass of the recording system cannot be reduced beyond certain limits. It is then possible that the subject will notice his movements through the strings which must be fastened on his head or neck. It seemed therefore necessary to use an optic system, which leaves the subject quite unaware of what is being recorded and uninfluenced by the recording. The method modified after Goldberg and described in this paper is very convenient in use and gives satisfactorily reproducible results.

When the influence of age on the amplitude of oscillations in our material is compared with that of Frucht no great difference is seen when the more limited age spectrum (20—70 years) of his series is taken into account. In the material of Frucht the measure of oscillations »Schwankungssumme» is in the age group of 60—70 years 1.21 times that one of the age group 20—30 years. In our series the corresponding ratio was 1.36 for the age groups 62—80 and 18—30 years. This is rather curious because in his method the experimental subjects were in an illuminated room facing a homogeneous white wall, eyes open and standing free (with boots on!) consequently not in the Romberg position. The absolute values of the amplitudes of swing and the »Schwankungssumme» are not directly comparable. The latter represents viz. the hypotenuse of a rectangular triangle with the lateral and the antero-posterior excursions as kathetes.

Frucht sees in the decrease of standing steadiness an indication of a general deterioration of the coordinative function of the central nervous system. Which component: the sense organs, the peripheral and central nervous structures or the musculature are thereby predominantly responsible, is not clear. It may be noted that indications of impairment practically of all the mentioned parts have been found to exist in old age. In weight discrimination experiments performed partly with the same people who acted as experimental subjects in this study a definite increase of the differential threshold was recorded (1). Likewise the vibratory (14) and corneal (7, 16) sensibility, hearing etc. are known to be impaired in old people. Pathological anatomical studies indicate rarefication of fibres in the spinal roots (13) etc. In view of all these facts the increase in the amplitude of swing which seems to be a regular event first in extreme old age i.e. in people over 80 years old, is not great. It is interesting to note that just beyond this age limit in weight discrimination experiments the proprioceptive, muscular mechanism tends to give way for the tactile, pressure component on which the perception of weight is then based (1). Whether this fact should be interpreted as an indication of the dominating role of proprioceptive mechanisms in normal standing steadiness, remains to be settled by more direct evidence. It is however well known that it cannot be solely responsible for standing steadiness, because anesthesia, iceing, of the foot sole leads to a positive Romberg test (5). As a test of biological age

the standing steadiness probably has only limited value. In the less advanced age of 60—70 years the deviations from the values found in young people are slight as a rule.

SUMMARY

1. Tests of standing steadiness with optic registration of the oscillations of the vertex were performed in 81 subjects aged 18—30 and 62—88 years.

2. The antero-posterior and the lateral oscillations were found to increase with age, the increase being most pronounced in people over 80 years old.

3. The lateral movement is usually less extensive than that occurring in the antero-posterior direction, the lateral swing being 71 per cent of the antero-posterior measure in young and 65 per cent in the old.

4. The mean extent of the antero-posterior diameter of the area of swing is 41.7 $\pm$ 1.6 in the young, 56.4 $\pm$ 2.8 in the 62—80 years old and 75.5 $\pm$ 6.3 in the over 81 years old.

REFERENCES

1. Boman, K., and E. Jalavisto: Acta physiol. scand. (In press.)
2. Fick, A.: Mechanische Arbeit und Wärmeentwicklung bei der Muskeltätigkeit. Leipzig 1882.
3. Frucht, Ad. H.: Z. Altersforschung 1952:*6:*129.
4. Goldberg, L.: Acta physiol. scand. 1943:*5:*Suppl. 16.
5. Heyd, W. H. S. C.: Quoted by Wiggers Physiology in Health and Disease. London 1944.
6. Jalavisto, E.: Duodecim 1952:*68:*1. (Finnish.)
7. Jalavisto, E., Orma and M. Tawast: Acta physiol. scand. 1951: *23:*224.
8. Lee, M. A. M., and N. Kleitman: Am. J. Physiol. 1923:*67:*141.
9. Liebert, H.: Arbeitsphysiologie 1941:*11:*151.
10. Miles, W. R.: J. industr. Hyg. 1922:*3:*316.
11. Monjé, M.: Luftf. med. 1942:*6:*132.
12. Murray, I. M.: J. Gerontology 1951:*6:*120.
13. Newman, H., and K. B. Corbin: Proc. Soc. exp. Biol. 1936:*35:*273.
14. Pearson, G. H. J.: Arch. Neurol. Psychiatr. 1928:*20:*482.
15. Vierordt, K.: Physiologie des Menschen, Tübingen, 1871. pp. 442 and 446.
16. Zobel, H.: Graefes Arch. Ophthal. 1938:*139:*667.